DESCRIPTION

PSEUDOTYPE RETROVIRAL VECTORS CONTAINING MEMBRANE PROTEINS HAVING HEMAGGLUTININ ACTIVITY

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Technical Field

The present invention relates to pseudotype viral vectors containing HN protein of paramyxovirus.

10   Background Art

Retroviral vectors have been used to express foreign genes in target cells for research, gene therapy, etc. Retroviral vectors can be produced with a relatively simple method, and also have some advantages, such as, to introduce foreign genes into the chromosomes of the host. Normally, viral proteins localized in the viral envelope play a crucial role in retroviral vector infection. Much effort has been expended to widen the range of host cells to which the vectors can infect or to develop viral vectors which infect only specific cells by modifying the envelope proteins of retroviral vector.

20   For example, a system, where VSV-G protein is integrated into the retroviral vector envelope, has been developed to ensure the infectivity to a broader range of host cells (H. Yu et al., 1999, Gene Therapy, 6, 1876-1883). VSV-G is a protein expressed on the surface of envelope of vesicular stomatitis virus, which is infectious to a considerably broad range of host cells. In addition, for example, Sendai virus F protein has been used as an envelope protein by way of experiment. An F protein-pseudotyped retrovirus was found to exhibit specific infectivity to asialoglycoprotein receptor-positive cells (M. Spiegel et al., 1998, Hepatology, 28, 1429-1429; M. Spiegel

30   et al., 1998, J. Virology, 72, 5296-5302□.

However, these conventional pseudotype retroviruses have only insufficient infectivity to various tissues and cells. For example, a variety of stem cells including hematopoietic stem cell can be important target cells in gene therapy or the like (Y. Hanazono,

35   Molecular Medicine, Vol. 36, No. 7, 1999), but most stem cells are in nondividing state (Abkowitz, J. L. et al., Nat Med, 2(2), 190-7,

1996). In general, it is difficult to introduce genes using the retroviral vector exhibiting low infectivity against such nondividing cells. Further, the vector system using conventional techniques has failed to introduce genes into extracellular matrix-abundant cells
5 such as lung airway mucosal epithelial cells. A method for introducing genes into these types of cells requires physically removing extracellular matrix, such as mucus, by washing. However, this method is complicated and tissues can be damaged.

10 Disclosure of the Invention

An objective of the present invention is to provide pseudotype retroviral vectors containing membrane proteins having hemagglutinin activity.

The present inventors selected proteins having hemagglutinin
15 activity as proteins to pseudotype retrovirus. Hemagglutinin (HAin) is a protein that induces hemagglutination (HA; erythrocyte agglutination), and a number of viruses are known to have this activity. The present inventors considered that a retroviral vector capable of high efficiency gene transfer to various types of cells and tissues
20 could be constructed based on a retrovirus with the envelope containing membrane proteins having hemagglutinin activity. In order to construct such a retrovirus, the present inventors used envelope proteins from paramyxovirus having a broad host range. First, based on a vector expressing the envelope protein of Sendai virus (SeV),
25 a viral vector derived from a mouse retrovirus was pseudotyped by SeV F and/or HN protein. Specifically, using murine stem cell virus (MSCV) as starting material, a retrovirus was constructed to have SeV F and/or HN proteins in addition to the ecotropic envelope protein or amphotropic envelope protein; the resulting vector was tested for
30 the efficiency of gene transfer to human cells (Examples 1 and 2).

When the packaging was carried out using the ecotropic envelope protein showing no infectivity to human cells, gene transfer to human cells was not achieved with the virus pseudotyped by F protein or HN protein alone. However, after being pseudotyped by co-expressing
35 F protein and HN protein, the virus, which originally had no infectivity to human cells, could introduce genes into human cells (Eco in Fig.

1). This result showed that the host range for infection of the viral vector could be widened by pseudotyping a vector derived from viruses other than Sendai virus using F protein and HN protein from Sendai virus.

5   It was found that, when the packaging was carried out using the amphotropic envelope protein having infectivity to human cells, the efficiency of gene transfer was markedly improved by pseudotyping the virus either by expressing HN protein alone or by co-expressing F protein and HN protein (Ampho in Fig. 1). The result demonstrated

10 that the retroviral vector could be pseudotyped by Sendai virus HN protein alone, and that the efficiency of gene transfer of retroviral vector could be improved either by pseudotyping using Sendai virus HN protein alone or by pseudotyping using Sendai virus F protein and HN protein.

15 The present inventors also prepared an amphotropic retroviral vector by pseudotyping with HN protein and determined the efficiency of gene transfer into human bone marrow cells including hematopoietic stem cells (Example 3). The vector pseudotyped by HN protein was infected to CD34-positive human bone marrow cells, and the cells

20 containing the introduced gene were fractionated by flow cytometry using CD34 as a marker. The result demonstrated that the pseudotyping using HN protein markedly improved the efficiency of gene transfer into both CD34-positive and CD34-negative cells (Fig. 3). The CD34-positive cell fraction is believed to contain hematopoietic stem

25 cells. Thus, the HN protein-based pseudotyping is also useful for gene transfer into hemocytes and hematopoietic cells including hematopoietic stem cells.

Then, using lentivirus which is expected to be more suitable as a vector for gene therapy as compared with various other types of

30 retroviruses, the present inventors constructed a vector pseudotyped by paramyxovirus F protein and/or HN protein. The lentivirus used is simian immunodeficiency virus (SIV) (Example 5), which offers various advantages including a higher degree of safety when compared with human immunodeficiency virus (HIV) commonly used in gene therapy.

35 SIV virus particles comprising Sendai virus (SeV) envelope protein were produced by preparing virosome containing the envelope

reconstituted from SeV or inactivated Sendai virus and fusing them with SIV pseudotyped by VSV-G protein. The virus particles were incubated with human cells to assess the efficiency of vector infection (Examples 6 and 7). The result demonstrated that SIV comprising the

5   envelope resulting from fusion with SeV envelope exhibited higher infectivity as compared with SIV pseudotyped by VSV-G protein (Fig. 8). The increased infectivity was attributable to the Sendai virus HN protein.

To precisely estimate the contribution of SeV envelope protein,

10   the present inventors prepared FHN virosome, F virosome, and HN virosome, and prepared vectors by fusing VSV-G-pseudotyped SIV with each virosome. Then, the resulting vectors were assessed based on infection experiments (Example 7). The vector that is fused with FHN virosome showed significantly higher efficiency of gene transfer than

15   that of SIV alone (Fig. 10). With HN virosome, the efficiency of gene transfer also improved. However, unlike FHN virosome, F virosome fusion did not result in any increase in the gene expression level. Thus, HN protein was demonstrated to be important to increase the efficiency of gene transfer.

20   Furthermore, using a SeV envelope protein expression vector, the present inventors produced a lentivirus vector which was pseudotyped by SeV envelope protein at the stage of packaging (Example 8). The SIV vector pseudotyped by F protein and HN protein in addition to VSV-G protein was produced at a higher titer. This viral vector could

25   be further enriched by centrifugation. The present inventors tested the vector in *in vivo* gene transfer assay.

Since conventional viral vectors hardly transfer genes into mucosal cells of tracheal epithelium, a pre-treatment to remove physical barriers are required to achieve gene transfer; for example,

30   without damaging cells by sulfur dioxide or the like, gene transfer at sufficiently high efficiency is not achieved by a gene transfer method using the VSV-G-pseudotyped HIV vector (L. G. Johnson et al., Gene Therapy, 7, 568-574, 2000). The present inventors tested whether the SIV vector pseudotyped by VSV-G protein, and Sendai virus F and

35   HN protein allowed efficient gene transfer into mucosal cells of tracheal epithelium without inflicting such damage (Example 9). The

above-mentioned pseudotype SIV vector for GFP expression was intranasally given to mice, and the expression of GFP protein was observed on tissue sections of trachea 3 days after administration; GFP fluorescence was detected in epithelial cells of the trachea (Fig.

5   11). Further, fluorescence signals resulted from GFP expression were also detectable in mucosal epithelium of septonasal mucus membrane and pseudostratified ciliated epithelium of the same individuals (Fig. 12). Thus, it was demonstrated that the viral vector of the present invention could introduce genes into cells having mucus, such as

10 mucosal epithelial cells, without damaging the cells or tissues.

Furthermore, the present inventors constructed an expression vector for a modified HN protein in which the cytoplasmic domain had been altered. Using this vector, they produced a novel pseudotyped viral vector. Specifically, they constructed vectors to express a

15 protein comprising the cytoplasmic domain of SIV envelope protein linked to SeV HN protein; a protein in which the cytoplasmic domain of SIV envelope protein had been substituted for the cytoplasmic domain of HN protein; and the SeV F protein in which the whole or a portion of the cytoplasmic domain had been deleted or replaced with the

20 cytoplasmic domain of SIV envelope protein; then, they further constructed pseudotyped SIV vectors containing these proteins (Examples 11 and 12). It was shown that these vectors were capable of transferring genes into human cells, including 293T cells and BEAS-2B cells. Thus, HN pseudotyped vectors were found to be capable

25 of transferring genes into human cells even in the absence of any other coexisting envelope proteins such as VSV-G. It was also shown that MSCV-based pseudotype retroviral vectors constructed using these expression plasmids for modified HN proteins were capable of transferring genes into human cells (Example 13). The pseudotyped

30 SIV vector containing modified HN protein was prepared on a large scale, enriched, and then allowed to infect human bone marrow cells. According to this result, the vector could transfer genes into CD34$^+$ bone marrow cells with high efficiency (Example 14).

Furthermore, the present inventors prepared HA-pseudotyped

35 lentivirus vector using the expression vector for the hemagglutinin (HA) protein of influenza virus. The resulting virus could introduce

genes into human cells and be highly enriched by centrifugation (Example 16). In addition, they prepared HA/HN-pseudotyped lentivirus vector containing influenza virus HA protein and modified HN protein of Sendai virus, and confirmed that the vector could transfer genes into human cells (Example 17).

As described above, the present inventors succeeded in constructing viral vectors ensuring gene transfer at a high efficiency using retroviruses pseudotyped by membrane proteins having hemagglutinin activity. Additionally, they demonstrated that the viral vectors could transfer genes at a high efficiency into hemocytes and hematopoietic cells including hematopoietic stem cells, and cells having mucus such as mucosal epithelial cells, in *ex vivo* or *in vivo* administration.

More specifically, the present invention relates to:

(1) a substantially pure pseudotype retroviral vector that comprises a membrane protein having hemagglutinin activity;

(2) the pseudotype retroviral vector according to (1), wherein the membrane protein having hemagglutinin activity is derived from a protein having hemagglutinin activity which is contained in a single-stranded negative strand RNA virus;

(3) the pseudotype retroviral vector according to (2), wherein the membrane protein having hemagglutinin activity is derived from HN protein of paramyxovirus and/or HA protein of orthomyxovirus;

(4) the pseudotype retroviral vector according to (2), wherein the membrane protein having hemagglutinin activity is a portion or the whole of a cytoplasmic domain of a protein having hemagglutinin activity which is contained in a single-stranded negative strand RNA virus, and wherein the portion or the whole has been modified by substitution, deletion, and/or addition;

(5) the pseudotype retroviral vector according to any one of (1) to (3), further comprising F protein of paramyxovirus;

(6) the pseudotype retroviral vector according to (5), wherein a portion or the whole of a cytoplasmic domain of the F protein has been modified by deletion and/or addition;

(7) the pseudotype retroviral vector according to any one of (1) to (6), further comprising an envelope protein derived from a virus

that is infectious to human cells;

(8) the pseudotype retroviral vector according to (7), wherein the vector comprises an amphotropic envelope protein derived from a retroviral;

(9) the pseudotype retroviral vector according to (7) or (8), wherein the vector comprises VSV-G protein derived from a vesicular stomatitis virus;

(10) the pseudotype retroviral vector according to any one of (1) to (9), wherein the retroviral vector is derived from oncovirus;

(11) the pseudotype retroviral vector according to any one of (1) to (9), wherein the retroviral vector is derived from lentivirus;

(12) the pseudotype retroviral vector according to (11), wherein the lentivirus is derived from simian immunodeficiency virus;

(13) the pseudotype retroviral vector according to (3) or (5), wherein the paramyxovirus is Sendai virus;

(14) the pseudotype retroviral vector according to any one of (1) to (13), wherein the vector comprises a foreign gene in an expressible manner;

(15) the pseudotype retroviral vector according to (14), wherein the vector is used for transferring genes into a cell having mucus;

(16) the pseudotype retroviral vector according to (15), wherein the cell having mucus is a mucosal epithelial cell;

(17) the pseudotype retroviral vector according to (16), wherein the mucosal epithelial cell is a mucosal epithelial cell of nasal cavity or pulmonary bronchial tube;

(18) the pseudotype retroviral vector according to (14), wherein the vector is used for transferring genes into a hemocyte or hematopoietic cell;

(19) the pseudotype retroviral vector according to (18), wherein the hemocyte or hematopoietic cell is a hematopoietic stem cell;

(20) a composition for gene transfer, the composition comprising the pseudotype retroviral vector according to any one of (14) to (19);

(21) the composition according to (20), wherein the composition is a pharmaceutical composition;

(22) a method for introducing a foreign gene into cells, the method comprising the step of contacting cells with the pseudotype retroviral

vector according to any one of (14) to (19);

(23) a packaging cell for producing the pseudotype retroviral vector according to any one of (1) to (19), the cell comprising, in an expressible manner, a DNA encoding a protein having hemagglutinin activity; and

(24) a method for producing the pseudotype retroviral vector according to any one of (1) to (19), the method comprising the step of transcribing a retrovirus-derived gene transfer vector DNA in the packaging cell according to (23).

The retroviral vector of the present invention is a substantially pure pseudotype retroviral vector pseudotyped by a membrane protein having hemagglutinin activity. As used herein, the term "viral vector" refers to a viral particle capable of transferring nucleic acid molecules into a host. The term "retroviral vector" refers to a vector comprising the retrovirus backbone. The term "having the retrovirus backbone" means that nucleic acid molecules in the viral particle constituting the vector are based on the retrovirus genome. For example, a vector in which the nucleic acid molecules in the virus particle contain the packaging signal sequence derived from the retrovirus genome is one of the retroviral vectors of the present invention.

The term "retroviral vector pseudotyped by membrane proteins having hemagglutinin activity" refers to a retroviral vector containing one or more membrane proteins having hemagglutinin activity which are not contained in the natural counterpart. As used herein, the term "substantially pure pseudotype retroviral vector" refers to the pseudotype retroviral vector which does not substantially have a replicative virus having viral hemagglutinin activity except retroviral hemagglutinin activity. A preferred pseudotype retroviral vector of the present invention is a vector having substantially no replicative virus except retrovirus. The term "replicative" means that a virus replicates and produces infectious virus particles in a host cell where the viral vector has been infected. For example, in cells where the pseudotype retroviral vector has been infected, when the HA titer representing hemagglutinin activity (erythrocyte agglutination) after vector infection is not

significantly elevated as compared with that at the early stage of infection, one can assess that the vector does not substantially have replicative virus containing membrane proteins having hemagglutinin activity.

5   The membrane protein having hemagglutinin activity may be a naturally occurring protein or an artificial protein; it is preferred that the protein has viral hemagglutinin activity. Various types of viruses have been reported to have hemagglutinin activity. The type of erythrocyte and the optimal reaction temperature to be used for

10   detecting the hemagglutinin activity depends on the specific virus type. It has also been reported that in the case of rubella virus the reaction requires the presence of calcium ion. In the case of arbovirus, the optimal pH of the reaction falls within a very narrow range. The viral hemagglutinin is present in the virion itself in

15   enterovirus or rubella virus, or present in smaller particles as well as in virion in arbovirus, adenovirus, etc. The poxvirus hemagglutinin is present as a non-virion particle containing lipids. The pseudotype retrovirus of the present invention may contain such proteins.   Type-III adenoviruses partially agglutinate rat

20   erythrocytes, which results in incomplete agglutination; such proteins can also be used as the membrane proteins having hemagglutinin activity.

The hemagglutinin activity (erythrocyte agglutination; HA titer) can be tested by a method known in the art (The society of research

25   associates of The National Institute of Health, Eds., General Experimental Virology, 2nd Ed, pp. 214-225, MARUZEN CO.). The erythrocytes include, for example, those from chicken (including chick and fowl), goose, rat, guinea pig, rhesus monkey, green monkey and human. The reaction temperature may be 0°C, 4°C, room temperature,

30   37°C, or the like, depending on the type of protein. Exemplary conditions of erythrocyte agglutination reaction for the respective viruses are shown below:

Table 1

Kind of virus			HA reaction		
			Erythrocyte	Temp.	pH
Adeno	Type I	3, 7, 11,14,16, 20,21,25,28	Rhesus monkey	37°C	Independent
	Type II	8, 9, 10,13,15, 17,19,22,23,24 26,27	Rat		
	Type III	1, 2, 4, 5, 6	Rat Incomplete agglutination		
Entero	Coxsackie	A 7	Chicken	Room temperature	Independent
		A 20, 21, 24	Human type O	4°C	
		B 1, 3, 5		37°C	
	Echo	3, 6, 7, 11, 12, 13, 19, 20, 21, 24, 29		4°C	
Reo		1	Human type O	Room temperature	Independent
		2			
		3	Cattle		
Myxo	Influenza	A	Chicken, human or guinea pig	Room temperature	7.2
		B			
		C	Chicken	4°C	
	Mumps		Chicken	Room temperature	
	Newcastle disease (NDV)				
	Para-influenza	1	Chicken or human	4°C	Independent
		2	Chicken		
		3	Human or guinea pig	4°C or room temp.	
	Measles		Green monkey	37°C	
Arbo			Goose or chick	37°C	Strictly acidic
Rhabdo	Rabies		Goose	0°C	6.4 strictly
	Vesicular stomatitis (VSV)				5.8 strictly
Pox	Vaccinia, Variola		Chicken	Room temp. or 37°C	Independent
Rubella			Chick or goose	4°C	6.2 or 7.2
Polyoma			Guinea pig	4°C	7.2
Rat (RV)			Guinea pig	Room temperature	7.2

Particularly preferred membrane proteins having hemagglutinin activity in the pseudotype retrovirus of the present invention are viral proteins; specifically, such proteins include HN proteins of

5   Paramyxovirus; HA proteins of orthomyxovirus and influenza virus; togaviral E1 protein; A27L, H3L, and D8L proteins of vaccinia virus; M and E proteins of flavivirus; E1 and E2 proteins of coronavirus; G1 protein of bunyavirus, etc. Among others, proteins from single-stranded negative strand RNA viruses are preferred, and HN

10   protein of paramyxovirus is particularly preferred, as a membrane protein having hemagglutinin activity to be contained in the pseudotype retrovirus of the present invention.

As used herein, the term "single-stranded negative strand RNA virus" refers to a virus whose genome comprises a single-stranded

15   negative strand (namely, (-) strand) RNA. Such viruses include paramyxovirus (Paramyxoviridae; including the genus Paramyxovirus, the genus Morbillivirus, the genus Rubulavirus, the genus Pneumovirus, and such), rhabdovirus (Rhabdoviridae; including the genus Vesiculovirus, the genus Lyssavirus, the genus Ephemerovirus, and

20   such), filovirus (Filoviridae), orthomyxovirus (Orthomyxoviridae; including Influenza viruses A, B, and C, Thogoto-like viruses, and such), bunyavirus (Bunyaviridae; including the genus Bunyavirus, the genus Hantavirus, the genus Nairovirus, the genus Phlebovirus, and such), arenavirus (Arenaviridae), etc.

25   As used herein, the term "paramyxovirus" refers to a virus belonging to the family of paramyxovirus (Paramyxoviridae). The paramyxovirus includes, for example, Sendai virus, Newcastle disease virus, Mumps virus, Measles virus, RS virus (Respiratory syncytial virus), rinderpest virus, distemper virus, monkey parainfluenza virus

30   (SV5), human parainfluenza viruses' type-1, -2, and -3, etc. Sendai virus includes wild-type strains, mutant strains, laboratory strains, artificially constructed strains, etc. Incomplete viruses such as DI particles (J. Virol. 68, 8413-8417(1994)), synthesized oligonucleotides, etc. can also be used as material to produce

35   vaccines of the present invention. HN protein is a protein of paramyxovirus virus. Known genes encoding paramyxovirus viral

proteins include NP, P, M, F, HN, and L genes. "NP, P, M, F, HN and L genes" of viruses belonging to the family Paramyxoviridae correspond to genes encoding nucleocapsid, phospho, matrix, fusion, hemagglutinin-neuraminidase and large proteins. Respective genes of
5   viruses belonging to subfamilies of the family Paramyxoviridae are represented in general as follows. NP gene is generally described also as the "N gene".

Genus Paramyxovirus	NP	P/C/V	M	F	HN	-	L
Genus Rubullavirus	NP	P/V	M	F	HN	(SH)	L
Genus Morbillivirus	NP	P/C/V	M	F	H	-	L

10   Database accession numbers for nucleotide sequences of genes of the Sendai virus classified into Paramyxovirus of the family Paramyxoviridae are, M29343, M30202, M30203, M30204, M51331, M55565, M69046 and X17218 for NP gene, M30202, M30203, M30204, M55565, M69046, X00583, X17007 and X17008 for P gene, D11446, K02742, M30202, M30203,
15   M30204, M69046, U31956, X00584 and X53056 for M gene, D00152, D11446, D17334, D17335, M30202, M30203, M30204, M69046, X00152 and X02131 for F gene, D26475, M12397, M30202, M30203, M30204, M69046, X00586, X02808 and X56131 for HN gene, and D00053, M30202, M30203, M30204, M69040, X00587 and X58886 for L gene.
20   As described herein in Examples, a retroviral vector pseudotyped by paramyxoviral envelope proteins can be produced by preparing inactivated paramyxovirus, virosome having the envelope protein of paramyxovirus, etc. and fusing it with retrovirus. The vector can also be produced by expressing an expression vector for the
25   paramyxovirus envelope proteins in retrovirus packaging cells.

The pseudotype retroviral vector of the present invention can comprise, for example, F protein of paramyxovirus in addition to membrane proteins having hemagglutinin activity such as HN protein of paramyxovirus. The present invention demonstrated that the
30   pseudotype retroviral vector containing HN and F proteins exhibited high efficiency of gene transfer. Pseudotype retroviral vectors containing the F and HN proteins are within the scope of the present invention. The pseudotype retroviral vector of the present invention

may further contain M protein of paramyxovirus.

Furthermore, the pseudotype retroviral vector of the present invention can contain additionally envelope proteins derived from other viruses. For example, preferred envelope proteins include those

5 derived from viruses infectious to human cells. Such proteins include, but not limited to, amphotropic envelope proteins of retrovirus, G protein of vesicular stomatitis virus (VSV), etc. Such proteins of viruses belonging to the herpes viridae include, for example, gB, gD, gH and gp85 proteins of herpes simplex virus, gp350

10 and gp220 proteins of EB virus, etc. Such proteins of viruses belonging to the hepadna viridae include S protein of hepatitis B virus, etc.

For example, a vector comprising retroviral amphotropic envelope (amphotropic env) protein and HN protein is preferred as a vector of the present invention. Alternatively, for example, the vector can

15 contain VSV-G protein that is a surface glycoprotein of vesicular stomatitis virus (VSV). VSV-G has been believed to use phospholipid as a receptor, which exists in most animal cells, and thus, by using a vector containing VSV-G protein and HN protein, the variety of cells into which genes can be introduced is dramatically increased and the

20 transfer efficiency is also elevated. Indeed, the vector containing VSV-G protein and HN protein exhibited higher efficiency of gene transfer than the vector containing VSV-G alone. Accordingly, the vector containing VSV-G protein and HN protein is a preferred vector of the present invention. Such vectors may further contain

25 paramyxovirus F protein. In summary, both vector containing retroviral amphotropic envelope protein, F protein, and HN protein, and vector containing VSV-G protein, F protein, and HN protein are within the scope of the present invention. In addition, these vectors may further contain M protein of paramyxovirus. Specifically, both

30 vector containing retroviral amphotropic envelope protein, F protein, HN protein, and M protein, and vector containing VSV-G protein, F protein, HN protein, and M protein are within the scope of the present invention. The vector containing paramyxoviral F and HN proteins, and the vector containing F, HN and M proteins, as described above,

35 also transferred genes at a high efficiency into cells having mucus, cell fractions containing hematopoietic stem cells, or the like, into

which genes were hardly transferred by the conventional techniques.

Since VSV-G protein is a glycoprotein and forms a stable homotrimer on the membrane, the vector particles are scarcely disrupted during purification; thus the particles can be concentrated to a high level by centrifugation (Yang, Y. et al., Hum Gene Ther: Sep, 6(9), 1203-13. 1995). The present inventors confirmed that the SIV vectors pseudotyped by VSV-G and by F and HN could be concentrated by centrifugation.

There is no limitation on the types of paramyxovirus HN, F, and M proteins to be used to prepare pseudotyped vectors. Particularly, the proteins of Respirovirus including Sendai virus are preferred. The HN, F, and M proteins of Sendai virus include, for example, those from Z strain. The retroviral amphotropic envelope proteins include, for example, the envelope proteins from mouse leukemia virus (MuLV) 4070A strain. The MuMLV 10A1-derived envelope proteins can also be used (for example, pCL-10A1 (Imgenex) (Naviaux, R. K. et al., J. Virol. 70: 5701-5705 (1996)). The ecotropic envelope proteins include, for example, the envelope proteins from Moloney murine leukemia virus (MoMuLV). Vesicular stomatitis virus G protein (VSV-G) includes, for example, the protein derived from Indiana serotype strain (J. Virology 39: 519-528 (1981)). In addition to these, the proteins may be derived from desired strains.

The above-mentioned envelope proteins, including HN, F, M, VSV-G, and retroviral envelope proteins, may be intact proteins from wild-type viruses or mutated proteins spontaneously or artificially. For example, HN protein, which is a structural protein, has both hemagglutinin (which is erythrocyte agglutination) activity and neuraminidase activity. For example, the reduction of the former activity may increase viral stability in blood. For example, the efficiency of infection via HN protein can be controlled by modifying the latter activity. Alternatively, the fusion efficiency can be controlled by modifying F protein that participates in the membrane fusion. Further, for example, the antigen presenting epitopes of F protein and HN protein that can serve as cell-surface antigen molecules are analyzed, and based on the analysis result, pseudotype retroviruses of the present invention can be prepared by using the

proteins whose antigen presenting ability has been impaired. In addition, it is possible to use the envelope proteins of attenuated strains of pathogenic paramyxoviruses.

For example, higher efficiency gene transfer vectors can be
5   prepared by pseudotyping the retroviral vector of the present invention by using modified proteins that are the viral envelope proteins having hemagglutinin activity or other envelope proteins whose cytoplasmic domains have been modified by deletion, substitution and/or addition. The present invention relates to pseudotype
10   retroviral vectors comprising proteins in which a portion or the whole of a cytoplasmic domain of wild-type membrane proteins having hemagglutinin activity has been modified by substitution, deletion, and/or addition. Specifically, for example, modified HN protein and/or F protein of paramyxovirus, in which the cytoplasmic domain has been deleted, or a cytoplasmic domain from another membrane protein
15   (for example, the envelope protein of retrovirus including lentivirus) has been substituted for or added to the original one, can be used preferably to produce a viral vector with high infectivity. The present invention provides a membrane protein having hemagglutinin
20   activity in which a portion or the whole of a cytoplasmic domain of the wild type of the membrane protein has been modified by substitution, deletion, and/or addition, and the nucleic acid encoding the protein (DNA, RNA, or the like). Particularly, the present invention provides a modified viral hemagglutinin protein whose cytoplasmic domain has
25   been modified by substitution, deletion, and/or addition, and the nucleic acid encoding the protein. For example, a modified HN protein comprising the amino acid sequence of SEQ ID NO: 40 or 41 is preferred. Such modified proteins and the nucleic acids encoding the proteins are useful to produce pseudotype viruses of the present invention.
30   Specifically, foreign genes can be introduced with high efficiency into a wide variety of cells including human cells, by using retrovirus pseudotyped with a modified paramyxovirus HN protein whose cytoplasmic domain has been replaced with the cytoplasmic domain of envelope protein of lentivirus such as SIV, or retrovirus (for example, the
35   protein encoded by pCAGGS-SIVct/HN according to Example 11); or another modified protein in which the cytoplasmic domain of envelope

protein of retrovirus such as lentivirus has been added to paramyxovirus HN protein (for example, pCAGGS-SIVct+HN according to Example 11). Arbitrary portions of the cytoplasmic domain of HN protein can be deleted; arbitrary portions of the cytoplasmic domain

5   of retroviral envelope protein can be added; a portion or the whole of cytoplasmic domain may be deleted, substituted, and/or added.

Such a viral vector can contain additionally a modified F protein of paramyxovirus. For example, it is possible to use an F protein of paramyxovirus whose cytoplasmic domain has been deleted, or a

10   protein obtained by adding the cytoplasmic domain of envelope protein of lentivirus such as SIV, or retrovirus to such truncated protein. Specifically, for example, a plasmid is constructed to express an F protein whose cytoplasmic domain amino acids are deleted. Any portions of the domain can be deleted; a portion or the whole of

15   cytoplasmic domain can be deleted. The pseudotype virus containing a truncated Sendai virus F protein (Fct4) that had only four amino acids of the cytoplasmic domain by deleting the remaining exhibited significantly high efficiency of gene transfer. Thus, the protein that contains none or only several amino acid residues of the

20   cytoplasmic domain due to an artificial deletion can be preferred to produce a pseudotype virus of the present invention. A protein in which the cytoplasmic domain of F protein has been substituted with a distinct peptide can be produced by attaching a portion or the whole of cytoplasmic domain of envelope protein of another virus

25   (for example, lentiviral envelope protein) to these truncated F proteins. For example, such proteins include the protein to which the first 11 amino acids of the cytoplasmic domain of SIV envelope protein from its 5' end (SIVct11) have been attached. Thus, the present invention provides a paramyxovirus F protein in which a portion or

30   the whole of cytoplasmic domain of the wild type of the protein has been modified by substitution, deletion, and/or addition, and the nucleic acid encoding the protein. In particular, the present invention provides an F protein whose cytoplasmic domain has been replaced with a portion of or the whole of a cytoplasmic domain of

35   the envelope protein from a retrovirus including lentivirus, and the nucleic acid encoding the protein. For example, a modified F protein

comprising the amino acid sequence of SEQ ID NO: 42, 43, 44, 45, 46, or 47 is preferred. These modified proteins and the nucleic acids encoding the proteins can be used to produce pseudotype viruses of the present invention.

5   It is also preferred that HA protein of a virus belonging to the Orthomyxoviridae is used as the viral envelope protein having hemagglutinin activity. For example, a pseudotype virus produced by using an expression plasmid for influenza virus envelope protein is infectious to a wide variety of mammalian cells including human cells.
10   The influenza virus envelope may be derived from a desired strain of isolated influenza virus. In the budding of influenza virus, neuraminidase is responsible for the cleavage of the linkage with sialic acid. Thus, infectious virus particles pseudotyped by HA can be prepared by the treatment with neuraminidase. Alternatively, the
15   linkage with sialic acid can be cleaved automatically by using a viral vector that also encodes a protein having neuraminidase activity. In such cases, it is particularly preferred to use a viral envelope protein having neuraminidase activity, such as HN protein of paramyxovirus. Thus, the present invention provides retroviral
20   vector pseudotyped by HA/HN protein.

    The retroviral vector of the present invention also includes those derived from oncovirus. The term "oncovirus" refers to retroviruses belonging to the oncovirus subfamily (Oncovirus). The oncovirus includes retroviruses involved in canceration, such as sarcoma virus,
25   leukemia virus, mammary tumor virus, etc. For example, Moloney murine leukemia virus (MoMLV) is one of the earliest developed retroviral vectors, which has a number of improvements and is widely used. A viral vector prepared by pseudotyping MoMLV with a protein having hemagglutinin activity, such as paramyxovirus HN protein, can be used
30   preferably in the present invention. In addition, murine stem cell virus (MSCV) used in Example is a preferred gene-transfer vector particularly in transferring genes into hemocytes, hematopoietic cells, embryonic stem cells, etc.

    The retroviral vector of the present invention also includes those
35   derived from lentivirus. The term "lentivirus" refers to a retrovirus belonging to the lentivirus subfamily (Lentivirus). The lentivirus

includes human immunodeficiency virus (HIV) (e.g., HIV1 or HIV2), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), Maedi-Visna virus, equine infectious anemia virus (EIAV), caprine arthritis-encephalitis virus (CAEV), etc. The retroviral
5 vector of the present invention can be derived from a desired strain or subtype. For example, HIV-1 includes those of every major (M) subtype (including A to J), N, and outlier (O) (Hu, D. J. et al., JAMA 1996; 275: 210-216; Zhu, T. et al., Nature 1998, 5; 391(6667): 594-7; Simon, F. et al., Nat. Med. 1998, 4(9): 1032-7). Isolated SIV
10 strains include, for example, SIVagm, SIVcpz, SIVmac, SIVmnd, SIVsnm, SIVsyk, etc.

Lentiviruses are infectious to nondividing cells and the virus genome can be integrated into host cell chromosome. The nuclear translocation signals encoded by gag and vpr are believed to be
15 responsible for the integration. When, using this characteristic, a viral vector of the present invention is constructed based on a lentivirus, genes can be introduced into nondividing cells in living tissues and cells that hardly divide, such as stem cells in various tissues, which allows long-term gene expression.

20 Human immunodeficiency virus (HIV), before any other lentivirus, was used to construct a vector, which can also be used preferably in the present invention. Vectors have been developed based on feline immunodeficiency virus (FIV) (Poeschla, E. M. et al., Nature Medicine, 4(3), 354-7, 1998) and caprine arthritis-encephalitis virus (CAEV)
25 (Mselli-Lakhal, L. et al., Arch. Virol., 143(4), 681-95, 1998). These vectors can be used to produce vectors of the present invention.

Simian immunodeficiency virus (SIV) was discovered as a monkey-derived HIV-like virus, which, along with HIV, forms the group of primate lentivirus (E. Ido and M. Hayamizu, "Gene, Infection and
30 Pathogenicity of Simian Immunodeficiency Virus", Protein, Nucleic acid and Enzyme, Vol.39, No.8, 1994). This group is further divided roughly into four subgroups: (1) HIV-1 subgroup containing HIV-1 that is the causative virus for human acquired immune deficiency syndrome (AIDS) and SIVcpz isolated from chimpanzee; (2) HIV-2 subgroup
35 containing SIVsmm isolated from Sooty Mangabey (*Cercocebus atys*), SIVmac isolated from rhesus monkey (*Macaca mulatta*), and HIV-2 that

is less pathogenic in human (Jaffar, S. et al., J. Acquir. Immune Defic. Syndr. Hum. Retrovirol., 16(5), 327-32, 1997); (3) SIVagm subgroup represented by SIVagm isolated from African green monkey (*Cercopithecus aethiops*); and (4) SIVmnd subgroup represented by

5 SIVmnd isolated from Mandrill (*Papio sphinx*).

There is no report suggesting the pathogenicity of SIVagm and SIVmnd in natural hosts (Ohta, Y. et al., Int. J. Cancer, 15, 41(1), 115-22, 1988; Miura, T. et al., J. Med. Primatol., 18(3-4), 255-9, 1989; M. Hayamizu, Nippon Rinsho, 47, 1, 1989). In particular,

10 previous reports describe that, according to the results of infection experiments, the TYO-1 strain, which is one of SIVagm and was also used herein in the Examples, is not pathogenic in crab-eating monkey (*Macaca facicularis*), rhesus monkey (*Macaca mulatta*) as well as in natural hosts (Ali, M. et al, Gene Therapy, 1(6), 367-84, 1994; Honjo,

15 S et al., J. Med. Primatol., 19(1), 9-20, 1990). There is no report on SIVagm infection to humans and the onset thereof, and thus it is believed that SIVagm may not be pathogenic to human. In general, lentiviruses in primate has strict species-specificity, and there are few reports on cases where cross-species infection with SIVagm

20 from natural hosts and onset thereof; if any, normally the onset frequency is low and the disease progresses slowly (Novembre, F. J. et al., J. Virol., 71(5), 4086-91, 1997). Accordingly, a viral vector prepared based on SIVagm, particularly SIVagm TYO-1, is thought to be safer than vectors based on HIV-1 or other lentiviruses, and thus

25 can be used preferably in the present invention.

Furthermore, the retroviral vector of the present invention includes those derived from spumavirus. The spumavirus includes, for example, foamyvirus (DE4318387; WO9607749; Virology (1995) 210, 1, 167-178; J. Virol. (1996) 70, 1, 217-22). Vectors of the present

30 invention derived from foamyvirus can be utilized for introducing foreign genes into human cells, particularly in gene therapy and administration of recombinant vaccines.

In the retroviral vector of the present invention, LTR (long terminal repeat) may be modified. LTR is a retrovirus-specific

35 sequence, which is present in the virus genome at both ends. 5' LTR serves as a promoter, which enhances the mRNA transcription from the

provirus. Thus, a substitution of the portion exhibiting the 5' LTR promoter activity in the gene transfer vector with another promoter having stronger promoter activity can lead to increased levels of mRNA transcription of the gene transfer vector, which may improve

5   the packaging efficiency and thus increase the vector titer. Furthermore, for example, in the case of lentivirus, the transcription activity of 5' LTR is known to be enhanced by viral tat protein, and therefore substitution of a tat protein-independent promoter for 5' LTR allows the exclusion of tat from the packaging vector. The

10   intracellular RNA of virus infected to cells is reverse transcribed and forms a closed circular structure with a linkage between the LTRs at the two ends, and then integrated into the chromosome of cells through the interaction between the linkage site and viral integrase. The mRNA transcribed from the provirus corresponds to the region from

15   the transcription initiation site in the 5' LTR to the 3' LTR polyA sequence that located downstream; the 5' LTR promoter portion is not packaged in the virus particle. Thus, even if the promoter is replaced with another sequence, the portion that is integrated into the chromosome of target cells has no alteration. Based on the facts

20   described above, a substitution of 5' LTR promoter can provide a safer vector with a higher titer. Thus, a substitution of the promoter at the 5' end in a gene transfer vector can increase the titer of packagable vectors.

The safety can be improved with a self inactivating vector (SIN

25   vector) which is prepared by partially eliminating the 3' LTR sequence to prevent the transcription of full-length vector mRNA in target cells thereof. The provirus for lentivirus, which is integrated into the chromosome of target cells, has the U3 portion of 3' LTR attached to the 5' end thereof. Thus, in the chromosome of target cells, the

30   gene transfer vector contains U3 at the 5' end and accordingly the transcription of RNA covering the whole gene transfer vector starts there. If there were lentivirus or related proteins in the target cells, the gene transfer vector would be re-packaged and infect other cells. Furthermore, there is a possibility that a host gene located

35   adjacent to the 3' end of virus genome may be expressed by the 3' LTR promoter (Rosenberg, N., Jolicoeur, P., Retroviral Pathogenesis.

Retroviruses. Cold Spring Harbor Laboratory Press, 475-585, 1997).
Such events are recognized as being problematic with retroviral
vectors. Thus, the SIN vector was developed as a solution to overcome
the problems (Yu, S. F. et al., Proc. Natl. Acad. Sci. U S A, 83(10),
3194-8, 1986). When the U3 portion is deleted from the 3' LTR in the
gene transfer vector, neither 5' LTR nor 3' LTR promoter is present
in the target cells. In such cases, neither full-length viral RNA
nor host gene is transcribed, while the transcription of genes of
interest is achieved with internal promoters; such a vector can be
an overexpression vector with higher safety, and thus is preferable
in the present invention. SIN vectors can be constructed according
to any of methods known in the art or the method as described in Example
5.

Retroviruses can be produced by transcribing a gene transfer vector
DNA containing the packaging signal in host cells, and allowing virus
particles formation in the presence of gag and pol proteins, and
envelope proteins. The gene transfer vector DNA may be a DNA vector
such as plasmid, or a DNA that has been integrated in the chromosome
of packaging cells. While it is preferable to integrate the packaging
signal sequence encoded by the gene transfer vector DNA as long as
possible to maintain the structure formed based on the sequence, it
is required to minimize the sequence homologous between the packaging
signal in the vector DNA and another packaging vector for providing
gag and pol proteins to reduce the frequency of wild-type virus
formation due to recombination between the types of vectors.
Accordingly, it is preferable to construct the gene transfer vector
DNA using as short a sequence as possible comprising the sequence
required for packaging to meet both criteria of packaging efficiency
and safety.

There is no limitation on the type of packaging signal, as long
as packaging is achieved in cells where the packaging vector has been
introduced; those derived from retrovirus, lentivirus,
immunodeficiency virus, and the like can be used depending on the
type of packaging vector.

For example, in the case of SIVagm-derived packaging vector used
in the Example, the type of virus from which the signal to be used

is derived is limited to SIV because HIV vectors are not packaged. However, the SIV-derived gene transfer vector is also packagable when an HIV-derived packaging vector is used. Thus, the frequency of recombinant virus formation can be reduced when the vector particles

5   are formed by combining gene transfer vector and packaging vector, each derived from different type of lentivirus. In such cases, it is preferred to use combinations of lentiviruses in primates (for example, HIV and SIV).

In a preferred gene transfer vector DNA, gag protein has been

10   modified so that it is not expressed. The viral gag protein can be detected as a foreign substance in the living body, and thus a potential antigen. Alternatively, the protein may affect cellular functions. To prevent the gag protein expression, frameshift mutations can be introduced for modification by adding or deleting nucleotides

15   downstream of the start codon of gag. It is also preferable to delete portions of the coding region of gag protein. In general, a 5′ portion of the coding region of gag protein is known to be essential for virus packaging. Thus, in a gene transfer vector, it is preferred that the coding region for the gag protein is deleted at the C terminus. It

20   is preferred to delete as large a portion of gag coding region as possible, as long as the deletion does not considerably affect the packaging efficiency. In addition, it is preferred to replace the start codon (ATG) of gag protein with a codon other than ATG. Such a codon for the replacement can be selected appropriately not to greatly

25   affect the packaging efficiency. A viral vector containing the transcription product of gene transfer vector DNA can be produced by introducing the constructed gene transfer vector DNA comprising the packaging signal into appropriate packaging cells. The viral vector particles produced can be recovered from the culture

30   supernatant of packaging cells, or the like.

There is no limitation on the type of packaging cell, as long as the cell line is generally used in viral production. When used for the purpose of gene therapy in human, a human or monkey-derived cell is suitable. Human cell lines to be used as packaging cells include,

35   for example, 293 cell, 293T cell, 293EBNA cell, SW480 cell, u87MG cell, HOS cell, C8166 cell, MT-4 cell, Molt-4 cell, HeLa cell, HT1080

cell, TE671 cell, etc. Monkey cell lines include, for example, COS1 cell, COS7 cell, CV-1 cell, BMT10 cell, etc. In addition, previously established packaging cells can be used. Such packaging cells include, for example, Bosc23 cell, PE501 cell, etc.

5   There is no limitation on the type of foreign gene to be inserted in the vector, which include, for example, nucleic acids which do not encode any protein, such as antisense or ribozyme, as well as protein-encoding nucleic acids.

     In recent years, attention is being given to a variety of stem
10 cells including hematopoietic stem cells as targets of gene therapy (Y. Hanazono, Molecular Medicine, Vol. 36, No. 7, 1999). As shown in Example, the pseudotype retroviral vector of the present invention can transfer genes into CD34-positive cells derived from human bone marrow with high efficiency; such a fraction comprising the
15 CD34-positive cells receives attention as a cell fraction containing hematopoietic stem cells in recent years. Previous reports describe that the CD34-positive cells exhibit pluripotency in colony assay using a culture medium containing methylcellulose (Kirshenbaum, A. S. et al., J. Immunol., 148(3), 772-7, 1992) and that transplantation
20 of CD34-positive cells into NOD/SCID mouse that is a compounded immunodeficiency strain leads to localization of the cells in the mouse bone marrow and reconstitution of hemopoietic system (Larochelle, A. et al., Nat. Med., 2(12), 1329-37, 1996). Hence, it is though that stem cell-like immature cells are present in at least
25 the CD34-positive cell fraction. The hematopoietic stem cells in the CD34-positive cell fraction are in nondividing state. In general, when a retroviral vector is used, the efficiency of gene transfer into such cells is low (Kiem, H. P. et al., Curr. Opin. Oncol., 7(2), 107-14, 1995.), but the infection efficiency can be greatly improved
30 by using the pseudotyped vector of the present invention. In particular, the efficiency of gene transfer into nondividing cells is expected to further increase by the use of a lentivirus vector, such as HIV or SIV vector. The present invention relates to a method for introducing genes into hemocytes or hematopoietic cells, in which
35 the method comprises a step of contacting pseudotype retroviral vector containing membrane proteins having hemagglutinin activity with

hemocytes or hematopoietic cells, and the use of the pseudotype retroviral vector containing membrane proteins having hemagglutinin activity to introduce genes into hemocytes or hematopoietic cells. The retroviral vector of the present invention, pseudotyped by the
5   use of proteins having hemagglutinin activity, can transfer genes into hemocytes and hematopoietic cells with high efficiency, and thus is useful in gene therapy whose target is blood cells, e.g., adenosine deaminase (ADA) deficiency (Blaese, R. M., Pediatr. Res., 33 (1 Suppl), S49-53, 1993), hemophilia (Kay, M. A. et al., Proc. Natl. Acad. Sci.
10   U S A, 96(18), 9973-5, 1999) and Fanconi anemia, etc. The administration can be performed, for example, by an *ex vivo* method.

Evaluation for the achievement of introduction of foreign genes into hemocytes and hematopoietic cells can be made, for example, by flow cytometry analysis using antibodies against various known surface
15   antigens or by colony assay, or through assessing hemopoietic cell transplantation-based reconstitution of hemopoietic system in mice whose hemopoietic system has been disrupted.

Gene therapy whose targets are hemopoietic cells, to which the vector of the present invention is applicable, includes, for example,
20   use of the drug resistance gene MDR1 to preserve stem cells in anti-cancer chemotherapy (Licht, T. et al., Gene Ther. (2000) 7, 4, 348-58); introduction of the normal FANCC gene for the treatment of Fanconi anemia (Liu, J. M. et al., Hum. Gene Ther. (1999) 10, 14, 2337-46); introduction of a combination of cytokines (thrombopoietin,
25   interleukins 6 and 11, and Flt-3 ligand) to enhance the *ex vivo* proliferation of stem cells (WO 9907831); the expression of chimeric proteins, such as Flt-3 agonist, to treat cytopenia (WO 9846750); introduction of the human β globin gene to treat β thalassemia (WO 9741141); a combination therapy with IL-6 antagonist and suicide gene
30   expression to treat IL-6-dependent multiple myeloma (German Patent No. DE19704979); introduction of genes such as receptor agonist comprising a combination of hemopoietic factors [interleukins (GM-CSF, G-CSF-Ser17, M-CSF, erythropoietin, IL-1, IL-14, IL-2, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, and IL-15),
35   leukemia inhibitory factor (LIF), flt3/flk2 ligand, human somatotropin, B cell growth factor, B cell differentiation factor,

erythrocyte differentiation factor (EDF) or stem cell factor (SCF)]
(WO 9712985), c-mpl receptor agonist to be used in stem cell culture
and gene therapy for hemopoietic diseases (WO 9712978), IL-6 and IL-6
soluble receptor fusion protein to be used for the proliferation of
5   human hemopoietic progenitor cells (Nat. Biotechnol. (1997) 15, 2,
142-45), IL-6 superagonist and superantagonist to be used to
proliferate hemopoietic progenitor cells (WO 9618648), Factor-X to
be used in therapy for blood diseases (J. Cell. Bioche. (1995) Suppl.
21A, 410), stem cell factor, IL-6, and soluble IL-6 receptor complex
10   to be used to proliferate human hemopoietic progenitor cells (Gene
Ther. (1995) 2, 9, 694), ribozyme whose targets are RNA viruses, and
antisense and/or decoy RNA which are useful to prevent HIV infection
and for intracellular immunity (WO 9622368).

The HN protein-pseudotyped retroviral vector of the present
15   invention is highly infectious to cells containing mucus, such as
mucosal epithelial cells of nasal cavity and epithelial cells of
bronchial mucus membrane in the lung. The vector of the present
invention is useful for high efficiency transfer of foreign genes
into cells containing mucus, into which genes are hardly transferred
20   by any of the conventional methods. The present invention relates
to a method for introducing genes into cells containing mucus, in
which the method comprises a step of contacting a pseudotype retroviral
vector containing membrane proteins having hemagglutinin activity
with cells containing mucus, and the use of the pseudotype retroviral
25   vector containing membrane proteins having hemagglutinin activity
to introduce genes into cells containing mucus. Such cells containing
mucus include mucosal epithelial cell in particular, specifically
for example, mucosal epithelial cells of nasal cavity or pulmonary
bronchial tubes.

30   Specific examples for the use include, for example, the induction
of immunoreaction through gene transfer (IL-2, IFN-γ, TGF-β, etc.)
into skin and mucous membrane where antigen presenting cells (APC)
are advantageously abundant (WO 9505853); vaccination against
rotavirus by oral administration of genes to mucous membrane (J. Virol.
35   (1998) 72, 7, 5757-61); mucosal administration to treat autoimmune
diseases (WO 9746253); mucosal administration to prevent infection

(WO 9621356); administration of genes to mucous membrane of female genital organs to prevent cancer of the cervix uteri caused by sex-transmitted diseases or papilloma virus infection (Infect. Immun. (1998) 66, 1, 322-29); and improvement of administration simplicity
5   and safety by administration *via* mucous membrane (Proc. Am. Assoc. Cancer Res. (1995) 36, 86 Meet., 418).

The pseudotype retroviral vector of the present invention can be prepared as a composition by appropriately combining with pharmaceutically acceptable carrier or medium. Specifically, for
10   example, the vector can be formulated as a composition appropriately in combination with sterilized water, physiological saline, culture medium, serum, phosphate-buffered physiological saline (PBS), etc. The composition may further contain other components including stabilizer, antimicrobial, etc. The composition of the present
15   invention can be in a dosage form such as aqueous solution, capsule, suspension, syrup, etc. A composition comprising the pseudotype retroviral vector of the present invention is useful as a reagent or a pharmaceutical. For example, a composition of the present invention can be used as a reagent for *in vitro* or *in vivo* gene transfer
20   into various cells, or as a pharmaceutical for *ex vivo* or *in vivo* gene therapy. In general, the administration to patients can be achieved, for example, by a method known to those skilled in the art, including intraarterial, intravenous, intraperitoneal, subcutaneous, enteral, oral, or intranasal administration, or *ex vivo*
25   administration. In particular, administration to mucous membrane of the nasal cavity or bronchial tubes, and *ex vivo* administration to hemocytes and hematopoietic cells are suitable.

The viral vector of the present invention can be used in gene therapy for various other genetic diseases. There is no limitation on the
30   type of disease to be treated. For example, diseases to be treated and the single causative genes thereof include: Gaucher disease, β -cerebrosidase (chromosome 20); hemophilia, blood coagulation factor VIII (X chromosome) and blood coagulation factor IX (X chromosome); adenosine deaminase deficiency, adenosine deaminase;
35   phenylketonuria, phenylalanine hydroxylase (chromosome 12), Duchenne muscular dystrophy, dystrophin (X chromosome); familial

hypercholesterolemia, LDL receptor (chromosome 19), cystic fibrosis, chromosomal translocation of CFTR gene. The targeted disease in which other multiple genes are thought to be involved include neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, ischemic encephalopathy, dementia, and intractable infection such as AIDS. A treatment to inactivate the HIV transcription factor may be considered, wherein an SIV based vector of this invention is worked *in vitro* into a hematopoietic stem cell removed from an AIDS patient extracellularly, for increasing the transcription of SIV-derived genome prior to HIV infection, and the transfected cell is returned to the patient's body. Furthermore, examples of applications possible for chronic diseases include: suppression of the expression of VEGF and FGF2 genes for ischemic heart disease, and suppression of the expression of cell proliferation related genes, such as cell proliferation factors (PDGF, TGF-β, etc.) and cyclin-dependent kinase, for gene therapy of arteriosclerosis. In addition, for diabetes, the BDNF gene may be a candidate. Furthermore, this method can be applied to substitution therapy, in which a gene such as a cancer suppressor gene, p53, whose genetic mutation causes canceration, is integrated into the chromosome, and this method enables treatment beyond the limitation of cancer pharmacotherapy by introducing a multiple-drug-resistant gene into bone marrow-derived hematopoietic stem cells *in vitro* and, then, by returning these cells into patient's blood. Regarding gene therapy of autoimmune diseases such as multiple sclerosis, chronic rheumatoid arthritis, SLE, and glomerulonephritis, expression suppression by antisense expression of T-cell receptors, various adhesion factors (for example, ICAM-1, LFA-1, VCAM-1, and LFA-4 etc.), cytokines and cytokine receptor (for example, TNF, IL-8, IL-6, and IL-1 etc.) cell proliferation factors (for example, PDGF, and TGF-β etc.), and activation factors (for example, MMP etc.) become possible. Regarding gene therapy of allergic diseases, expression suppression by antisense expression of IL-4, FcϵR-I, and such becomes possible. Regarding gene therapy relating to organ transplantation, the elevation of success percentage of a xenotransplant becomes possible by changing the histocompatibility antigen of a non-human animal donor to a

human-type. Furthermore, treatment by introducing foreign genes into the chromosome of human ES cells, thus making up the deficient genes at the embryonic stage to supplement deficiencies of systemically circulating enzymes, growth factors, and such may be considered.

5   For example, IL-4 promotes differentiation of helper T lymphocyte into Th2 lymphocyte. Th2 lymphocyte secretes cytokines, such as IL-4, IL-5, IL-9, and IL-13, which mediate asthmatic inflammation. IL-4 is a molecule that induces mucus secretion from lung mucus membrane, which is involved in respiratory disturbance. IL-4 regulates the
10   expression of VCAM-1 that is a cell adhesion molecule interacting with VLA 4 molecule present on the surface of eosinophil. The interaction allows eosinophils to migrate from blood to inflammation sites in lung tissues. Since IL-4 increases the number of B cells and induces the production of antigen-specific IgE responsible for
15   allergic reactions. The antigen-specific IgE produced induces the release of inflammatory mediators, such as histamine, leukotriene, from mast cells, which results in bronchoconstriction. Based on such role of IL-4, expression vectors for soluble interleukin 4 (IL-4) receptor etc. can be used to treat asthma patients.
20

Brief Description of the Drawings

     Fig. 1 depicts photographic patterns showing the results obtained with 293T cells infected with murine stem cell virus (MSCV) pseudotyped by F, HN, or F and HN proteins from Sendai virus. The virus packaging
25   was achieved by using a gene transfer vector expressing EGFP (pMSCV EGFP). The labels in the above panels, "F", "HN", and "F/HN", correspond to the results obtained by the infection with the viruses produced by expressing F, HN, and F and HN proteins of Sendai virus in the packaging cell, respectively. In the column of "VSV-G/Null",
30   the top panel depicts a positive control pseudotyped by VSV-G; the middle and bottom panels depict non-pseudotyped negative controls without expressing env proteins for pseudotyping, such as F, HN, and VSV-G. The labels at the right of the panels indicating "Eco" and "Ampho," correspond to the results obtained by using viruses prepared
35   by expressing ecotropic env and amphotropic env in the packaging cells, respectively. The label "Null" indicates that retrovirus env was not

expressed.

Fig. 2 depicts a photographic pattern showing results of HA assay of retroviruses having amphotropic env, which were pseudotyped by Sendai virus HN. The amounts of pMSCV EGFP, pCl-Ampho, and pCAGGS-HN used in viral production were also indicated (MSCV:AMPHO:HN in this Figure). While erythrocyte agglutination was not detected with the control retrovirus containing amphotropic env (Ampho-Retro in this Figure), the agglutination was observed with the retrovirus pseudotyped by Sendai virus HN (HN-Ampho-Retro in this Figure).

Fig. 3 is a diagram showing a result of assay which comprises infecting amphotropic env-containing MSCV pseudotyped by HN protein of Sendai virus (HN-ampho in this Figure) to human bone marrow cells and measuring the percentage of infected cells (GFP-expressing cells) by flow cytometry-based fractionation using CD34 as a marker. The "ampho" indicated in this Figure refers to a control which is not pseudotyped by HN protein. Each column indicates the ratio of GFP-positive cell against CD34-negative (CD34-) cell or CD34-positive (CD34+) cell.

Fig. 4 depicts a schematic illustration of structures of: (a) the SIVagm-genome plasmid which was used as the backbone for the other constructs, (b) constructed packaging vector, (c) gene transfer vector, and (d) VSV-G-supplying vector.

Fig. 5 depicts photographs showing a result of SIVagm vector-mediated introduction of the β-galactosidase gene into 293T cells. The top panel shows a result obtained by X-gal staining 48 hours after vector infection into 293T cells using the supernatant of cells transfected with the vector plasmid. The expression of β-galactosidase is detected in a number of cells. The bottom panel shows X-gal staining of untreated control cells.

Fig. 6 depicts photographs obtained by introducing the EGFP gene into G2-M phase-arrested 293T cells and SH-SY5Y cells differentiated by retinoic acid, in which gene transfer was mediated by the SIVagm SIN vector, and observing the resulting expression using a fluorescent microscope. The expression was confirmed under a fluorescence microscope. The top panel, 293T, magnification = x 100; the bottom panel, SH-SY5Y, magnification = x 200.

Fig. 7 is a photograph showing a result of Western blotting using FHN virosome and F virosome arranged in approximately the same particle diameter.

Fig. 8 depicts photographs showing a result obtained by the observations of infected cells (GFP-expressing cells). SIVs fused with inactivated Sendai virus (SeV), FHN virosome, and F virosome (respectively, corresponding to SIV-SeV fusion, SIV-FHN virosome fusion, and SIV-F virosome fusion in this Figure) were added to the culture supernatant of HeLa cells at MOI=10. The mixtures were incubated for 10, 30, and 180 minutes for infection, and then the culture media were changed with fresh ones. The cells were observed 48 hours after the infection. The SIV used had been pseudotyped by VSV-G. SIV in this Figure refers to a negative control which was not fused with Sendai virus envelope.

Fig. 9 depicts photographs showing a result obtained by electrophoresing samples of FHN virosome, F virosome, and HN virosome, followed by silver staining. Lane 1, SeV (intact); lane 2, DTT-treated SeV; lane 3, trypsin-treated SeV; lane 4, FHN virosome; lane 5, F virosome; lane 6, HN virosome.

Fig. 10 depicts a diagram showing a result of assay for relative LacZ activity. A LacZ expression SIV vector (pseudotyped by VSV-G) and the same but further fused with virosome were introduced into 293T cells. Asterisk indicates that there was a significant difference for SIV in t-test (p<0.05).

Fig. 11 depicts photographs showing frozen sections of trachea three days after intranasal administration of 100 μl of SIV-F/HN/M-EGFP 10^8 T.U. The arrows in (b) indicate epithelial cells of the trachea, where fluorescence of EGFP can be detected, while only low-level background signals are observed in the sample of the untreated mouse shown in (c). The panel (a) shows a pattern of hematoxylin-eosin (H.E.) staining of a serial section.

Fig. 12 depicts photographs (b) showing observations in septonasal mucus membrane of the same individuals as in Fig. 11. Arrow indicates pseudostratified ciliated epithelium that exhibits fluorescence of EGFP. (a) depicts photographs of H.E.-stained serial sections.

Fig. 13 depicts photographs showing a comparison of fluorescence

intensities of EGFP from the pseudotype retroviral vector in the sections of mouse nasal cavity. EGFP fluorescence images of SIV pseudotyped by VSV-G (SIV-VSV-EGFP), MSCV containing amphotropic env pseudotyped by F, HN, and M (MSCV-F/HN/M-EGFP), and VSV-G SIV

5   pseudotyped by F, HN, and M (SIV-F/HN/M-EGFP) are shown. Relatively stronger signals are detected in MSCV-F/HN/M-EGF and SIV-F/HN/M-EGFP containing F, HN, and M proteins; particularly, the signal is higher in SIV-F/HN/M-EGFP.

Fig. 14 shows the amino acid sequence (SEQ ID NO: 40) at the boundary
10   between the SIV cytoplasmic domain and the HN protein transmembrane domain (standard font) in the protein encoded by the HN expression plasmid with a substitution of cytoplasmic domain.

Fig. 15 shows the amino acid sequence (SEQ ID NO: 41) at the boundary between the cytoplasmic domain (underlined) and the HN protein
15   transmembrane domain (standard font) in the protein encoded by the HN expression plasmid with an addition of SIV cytoplasmic domain.

Fig. 16 shows the amino acid sequences (SEQ ID NOs: 42 to 44) at the boundary between the F protein transmembrane domain (italic) and F protein cytoplasmic domain (standard font) in the proteins encoded
20   by the F expression plasmids with a deletion of cytoplasmic domain.

Fig. 17 shows the amino acid sequences (SEQ ID NOs: 45 to 47) at the boundaries between the F protein transmembrane domain (italic without underline), F protein cytoplasmic domain (standard font), and the 11 amino acids of SIV cytoplasmic domain (SIV_{c11}) (underline)
25   in the proteins encoded by the F expression plasmids in which the F cytoplasmic domain has been replaced with the SIV cytoplasmic domain.

Fig. 18 depicts photographs showing gene transfer into 293T cells via the SeV F/HN pseudotype SIV vector.

Fig. 19 depicts photographs showing gene transfer into BEAS-2B
30   cells via the SeV F/HN pseudotype SIV vector.

Fig. 20 depicts photographs showing gene transfer into 293T cells via the SeV F/HN pseudotype SIV vector with an addition of SIV_{c11}.

Fig. 21 depicts photographs showing the enriched SeV F/HN pseudotype SIV vector.

35   Fig. 22 depicts photographs showing gene transfer via the MSCV-based SeV F/HN pseudotype retroviral vector.

Fig. 23 depicts photographs showing gene transfer *via* the viral vector pseudotyped by influenza virus envelope.

Fig. 24 depicts photographs showing the enriched viral vector pseudotyped by influenza virus envelope.

Fig. 25 depicts photographs showing gene transfer *via* viral vectors pseudotyped by influenza virus envelope and various HN proteins.

Best Mode for Carrying out the Invention

The present invention will be specifically described below using Examples; however, it is not to be construed as being limited thereto. All publications cited herein are incorporated herein by reference in their entireties.

[Example 1] Preparation of retroviral vectors pseudotyped by Sendai virus envelope protein

The genes for F, HN, and M proteins were obtained by digesting the full-length genome DNA of Sendai virus Z strain pSeV18$^+$b(+) (Hasan, M. K. et al., 1997, J. General Virology 78: 2813-2820), and inserted into pCAGGS (Niwa, H. et al., Gene: 108, 193-9, 1991) at the *Xho*I site, to prepare expression vectors for F, HN, and M proteins of Sendai virus (referred to as pCAGGS F, pCAGGS HN, and pCAGGS M, respectively).

The human fetal kidney-derived cell line, 293T cell (Proc. Natl. Acad. Sci. USA, vol.90, pp. 8392-8396, 1993), which was used to produce retrovirus, was cultured in D-MEM (GibcoBRL) containing 10% inactivated fetal bovine serum. Culture was carried out in plastic plates (Sumitomo Bakelite). The vector transfection was carried out using LIPOFECTAMINE PLUS (GibcoBRL) according to the provided instruction. 293T cells were plated in a 6-well plastic plate (Sumitomo Bakelite) at a cell density of 1x 10^6 cells/well, and incubated in a CO_2 incubator under 10% CO_2 gas at 37°C for 48 hours. 30 minutes before transfection, the culture medium was changed with 800 µl/well of D-MEM (GibcoBRL) containing 1% bovine serum albumin (GibcoBRL), and then culture was continued.

The gene transfer vector used was a murine stem cell virus-based vector (MSCV) (CLONTECH) (R. G. Hawley et al., Proc. Natl. Acad. Sci.

USA 93: 10297-10302 (1996); R. G. Hawley et al., Gene Thrapy 1: 136-138 (1994)) containing the EGFP gene as a reporter gene. Amounts of DNAs used in the transfection are as follows: 700 ng/well of the gene transfer vector and 300 ng/well of packaging vector (pCL-Eco,

5   pCL-Ampho (MuMLV 4070A) (both were purchased from IMGENEX)(Naviaux, R. K. et al., J. Virol. 70: 5701-5705 (1996)); these were used in combination with 200 ng/well expression vector for Sendai virus envelope protein (pCAGGS F and pCAGGS HN) and 200 ng/well expression vector for Sendai virus M protein (pCAGGS M) (Table 2). DNA was

10 dissolved in 100 μl of OptiMEM and then 6 μl of PLUS reagent (GibcoBRL) was added thereto. The mixture was stirred and allowed to stand still at room temperature for 15 minutes. 4 μl of LIPOFECTAMINE was diluted with 100 μl of OptiMEM, and then added to the mixture of DNA and PLUS reagent (GibcoBRL). The resulting mixture was stirred and allowed

15 to stand still at room temperature for 15 minutes. The solution containing the complex of DNA and LIPOFECTAMINE prepared by the above procedure was added dropwise to 293T cells incubated in a 6-well plate. After being gently stirred, the mixture was incubated in a CO_2 incubator under 10% CO_2 gas at 37°C for 3 hours. After culture, 1 ml/well of

20 D-MEM (GibcoBRL) containing 1% bovine serum albumin (GibcoBRL) and 15 μg/ml trypsin (GibcoBRL) was added to the culture. The mixture was incubated in a CO_2 incubator under 10% CO_2 gas at 37°C for 24 hours. Then, the culture supernatant was filtered with a filter with 0.45-μm diameter pores (DISMIC-25CS filter; ADVANTEC). The resulting

25 solution was used as a vector solution.

[Example 2]   The effect of Sendai virus envelope protein on pseudotyping of retrovirus vector

    The retroviral vector pseudotyped by Sendai virus envelope protein

30 was prepared by the above-mentioned method and its effect was assessed.

    293T cells used as a target were plated in a 6-well plastic plate (Sumitomo Bakelite) at a cell density of 1x 10^6 cells/well, and then incubated in a CO_2 incubator under 10% CO_2 gas at 37°C for 48 hours. The introduction of the viral vector into the target cells was achieved

35 by overlaying the solution that had been prepared by adding inactivated fetal bovine serum and polybrene (Sigma) at the final concentrations

of 10% and 8 µg/ml, respectively, to a solution containing the viral vector. 48 hours after vector introduction, the target cells were fixed using PBS (Invitrogen) containing 2% formaldehyde and 0.2% glutaraldehyde at room temperature for 20 minutes, and then washed

5 once with PBS (Invitrogen). Then, the cells were observed under a fluorescence invert microscope (DMIRB(SLR), Leica) to detect EGFP expression in target cells.

First, either or both F protein expression plasmid (pCAGGS F) and HN protein expression plasmid (pCAGGS HN) which are Sendai virus

10 envelope proteins, were co-introduced with a retrovirus-based gene transfer vector (pMSCV EGFP) into 293T cells in the absence of packaging vector. The culture supernatant was collected and tested for the introduction of the EGFP gene or EGFP protein into 293T cells as target cells. Results obtained by infecting 293T cells with the vectors

15 prepared by using in various combinations the 12 types starting from "((1))" in the lower half of Table 2 are shown in Fig. 1. As shown in the panel "Null" in Fig. 1, in the absence of packaging vector, neither gene nor protein was transferred, even when F protein and HN protein were expressed in human cells. Thus, the expression of

20 F protein and HN protein alone was found to be insufficient to produce retroviral vectors capable of transferring genes and to introduce EGFP protein into target cells.

When packaging is carried out using the ecotropic envelope protein that is an envelope protein having no infectivity to human cells,

25 gene transfer into human cells was not achieved with the vector pseudotyped by F protein or HN protein alone, as shown in the panel "Eco" in Fig. 1. On the other hand, the vector pseudotyped by co-expressing F protein and HN protein transferred the gene into human cells to which the original virus is not infectious. The result

30 indicates that vectors derived from viruses other than Sendai virus can be pseudotyped by Sendai virus F protein and HN protein, and thus the host ranges for infection of vectors other than Sendai virus can be widened.

When packaging was carried out using the amphotropic envelope

35 protein having infectivity to human cells, the efficiency of gene transfer improved by the vector pseudotyped either by expressing HN

protein alone, or by co-expressing F protein and HN protein, as seen in the panel "Ampho" in Fig. 1. The result indicates that vectors derived from viruses other than Sendai virus can be pseudotyped by Sendai virus HN protein alone, and the efficiency of gene transfer

5 mediated by a vector derived from a virus other than Sendai virus can be improved by pseudotyping the vector either with Sendai virus HN protein alone, or with Sendai virus F protein and HN protein.

In addition, since typically Sendai virus has high infectivity to cells containing mucus, such as epithelial cells of bronchial mucus

10 membrane in the lung, other vectors pseudotyped either by HN protein alone, or by F protein and HN protein can be used for gene transfer into cells containing mucus.

[Example 3] Production of HN-Ampho pseudotype retroviral vector and

15 comparison of the efficiency of gene transfer into human bone marrow cells including hematopoietic stem cells between the pseudotype viral vector and the amphotropic retroviral vector

1. Culture of 293T cells

293T cells were cultured in Dulbecco's modified Eagle medium (DMEM)

20 containing 10% FCS and 800 μg/ml G418 according to the conventional method. Using a 10-cm plate, the cells diluted to $8x\ 10^6$ cells/plate the day before transfection, and then cultured in DMEM containing 10% FCS.

2. Transfection

25 5.6 μg of pMSCV EGFP, 2.4 μg of pCl-Ampho (IMGENEX), and 1.6 μg of pCAGGS-HN were combined together, and 800 μl of OPTIMEM was added thereto. 48 μl of the Plus solution in a Lipofectamine Plus kit (GIBCOBRL) was further added to the mixture, and the resulting mixture was allowed to stand still for 15 minutes. In a separate tube, 800

30 μl of OPTIMEM and 32 μl of Lipofectamine solution combined together. The DNA mixture and Lipofectamine mixture were mixed and allowed to stand still for 15 minutes. The culture medium was removed and the transfection mixture was added dropwise all over to 293T cells in 500 μl of DMEM containing 1% BSA. The cells were incubated under

35 5% CO_2 gas at 37°C. A control transfection experiment was carried out using 5.6 μg of pMSCV EGFP and 4.0 μg of pCl-Ampho. Three hours

after transfection, the culture medium was replaced with 10 ml of Iscove's modified DMEM (IMDM) containing 10% FCS. On the following day, the medium was again changed with IMDM containing 10% FCS, and the culture was further continued for 24 hours.

5   3. Collecion of virus solution

The cell culture supernatant was collected 48 hours after transfection. The supernatant was filtered with a 0.45-μm filter and then stored at -80°C.

4. Assay for hemagglutinin activity

10   Retroviruses having amphotropic env pseudotyped by HN were prepared at various ratios of pMSCV EGFP, pCl-Ampho, and pCAGGS-HN in the same manner as described above. The viral hemagglutinin activity (erythrocyte agglutination activity; HA activity) was tested. The hemagglutinin activity of retrovirus having amphotropic

15   env was also tested as a control. Assay for HA activity was carried out according to the conventional method. The result showed that the retrovirus pseudotyped by HN and amphotropic env had HA activity and the virus particles contained proteins exhibiting hemagglutinin activity (Fig. 2).

20   5. Gene transfer into human bone marrow CD34$^+$ cells

Human bone marrow CD34$^+$ cell was purchased from BIO WHITTAKER. After thawing, along with 1x 10^5/ml of the recombinant virus collected in Section 3, the cells were cultured in IMDM containing 50 ng/ml IL-6, 100 ng/ml TPO, 100 ng/ml Stem Cell Factor (GIBCO BRL), 100 ng/ml

25   Flt-3 ligand (Research Diagnostics, Flanders, NJ) (all the above were human recombinant proteins), and 10% FCS under 5% CO$_2$ at 37°C overnight. 48, 51, 72, and 75 hours after the start of culture, the medium was changed with a freshly thawed virus solution, and 50 ng/ml IL-6, 100 ng/ml TPO, 100 ng/ml Stem Cell Factor, and 100 ng/ml Flt-3 ligand

30   were added to the cells. 120 hours after the start of culture, the cells were collected.

6. Analysis by flow cytometry

The collected cells were stained with PE-labeled anti-human CD34 antibody (Becton Dickinson), and then analyzed by flow cytometry

35   (EPICS ELITE, Coulter) using two fluorescence probes of GFP and PE. According to the result, the proportions of GFP positive cells in

CD34-negative cells (CD34-) and CD34-positive cells (CD34+) infected with the amphotropic retroviral vector (ampho) containing the wild-type envelope were only 0.3% and 3.6%, respectively. However, the proportions of GFP positive cells in CD34-negative cells (CD34-)

5 and CD34-positive cells (CD34+) infected with the HN-amphotropic retrovirus vector (HN-ampho) containing the pseudotype envelope were 33% and 25%, respectively, and thus the pseudotyping with HN protein was found to significantly increase the efficiency of gene transfer into human bone marrow cells including hematopoietic stem cells (Fig.

10 3).

[Example 4] Method for preparing pseudotype virus vectors

Retroviral vectors pseudotyped by various envelope proteins were prepared using the above-described pMSCV EGFP or pLZRNL expressing

15 lacZ under the control of LTR derived from Moloney murine sarcoma virus (Yee, J.-K. et al., Methods In Cell Biology, vol. 43, pp.99-112 (1994); Xu, L. et al., Virology 171, 331-341 (1989)) as a gene transfer vector.

293T cells (human fetal kidney cell line) were cultured in

20 Dulbecco's Modified Eagle Medium (DMEM)-High glucose (Gibco BRL) containing 10% inactivated calf serum (BIO WHITTAKER) under 10% CO_2 at 37°C. The 293T cells were plated in a 6-well plastic culture plate at a cell density of 5x 10^5 cells/well, and then incubated under 10% CO_2 at 37°C for 48 hours. The culture medium was changed with 800

25 µl/well of DMEM containing 1% bovine serum albumin. The cells were then used in transfection. In the combinations as indicated in Table 2 below, 700 ng of gene transfer vector (pMSCV EGFP or pLZRNL), 100 ng of VSV-G expression plasmid pVSV-G (derived from Indiana serotype strain) (Clontech), 200 ng each of Sendai virus HN, F, and M protein

30 expression plasmids, pCAGGS-HN, pCAGGS-F, and pCAGGS-M, and 300 ng each of mouse retrovirus coat protein expression plasmids, pCL-Eco and pCL-Ampho (Imgenex)(Naviaux, R. K. et al., J. Virol. 70: 5701-5705 (1996)), were dissolved in 100 µl of Opti MEM in each well. Then, 6 µl of PLUS Reagent (Gibco BRL) was added to the well. The mixture

35 was stirred, and then allowed to stand still at room temperature for 15 minutes. A solution obtained by diluting 4 µl of LIPOFECTAMINE

Reagent (Gibco BRL) with 100 µl of Opti MEM was added to the mixture. The mixed solution was stirred, and then allowed to stand still at room temperature for another 15 minutes. The resulting mixture was added dropwise to the 293 T cells prepared above while being stirred

5   gently. The cells were then incubated under 10% CO_2 at 37°C for 3 hours. 1 ml of DMEM containing 1% bovine serum albumin and 15 µg/ml trypsin (Gibco BRL) was added to each well. The cells were incubated under 10% CO_2 at 37°C for 48 hour, and then the culture supernatant was collected, and filtered with a filter with 0.45-µm diameter pores;

10 the resulting solution was used as a vector solution.

Table 2

	env		Gene transfer vector	
	Retro	SeV/VSV	in vitro	in vivo
①		F/M	pMSCV-EGFP or pLZRNL	pMSCV-EGFP
②	-	HN/M		
③		F/HN/M		
④		F/M		
⑤	Eco	HN/M		
⑥		F/HN/M		
⑦		F/M		
⑧	Ampho	HN/M		(⑨ : Fig. 13)
⑨		F/HN/M		
⑩	Eco	-		
⑪	Ampho	-		
⑫	Eco	VSV-G		
(①)		F	pMSCV-EGFP or pLZRNL (Fig.1)	ND
(②)	-	HN		
(③)		F/HN		
(④)		F		
(⑤)	Eco	HN		
(⑥)		F/HN		
(⑦)		F		
(⑧)	Ampho	HN		
(⑨)		F/HN		
(⑩)	Eco	-		
(⑪)	Ampho	-		
(⑫)	Eco	VSV-G		

(Those indicated in the column "Env" in this Table are envelope proteins derived from retrovirus (Retro), Sendai virus (SeV), and vesicular stomatitis virus (VSV) used in the production of viruses. The gene transfer vector used were pMSCV EGFP or pLZRNL, which were utilized in *in vitro* and *in vivo* gene transfer, as shown in this Table. The vector indicated in the upper (9) in the Table, which had been prepared using pMSCV EGFP as a gene transfer vector, was used in the experiment in Fig. 13. The 12 types of vectors indicated in lower (1) to (12), which had been prepared using pLZRNL, were used in the experiment in Fig. 1.)

[Example 5] Construction of VSV-G pseudotype lentivirus vectors

A nonpathogenic clone of African green monkey immunodeficiency virus, SIVagm TYO-1 strain, was used to construct vectors.

Hereinafter, nucleotides are numbered while taking the transcription
initiation site of viral RNA as +1. A SIVagm TYO-1-inserted plasmid
used was pSA212 (J. Viol., vol.64, pp.307-312, 1990). Every ligation
reaction was carried out using Ligation High (Toyobo) according to

5   the attached instruction. All synthetic oligonucleotides used were
synthesized and purified either with reverse-phase cartridges or by
reverse-phase HPLC in the Biochemical Research Division of the Nippon
Flour Mills Co., Ltd. *via* the Department of Custom DNA Synthesis,
Greiner Japan. The sequence of the synthetic oligonucleotides are

10   as follows:

     1F(VTRF-BglII):

5'-GCAGATCTCAACCAGGAGGCGAGGCTGCATTTTGGG-3' (SEQ ID NO: 1)

     1R(VTRR-EcoRI):

5'-GCGAATTCTACTTACTGGTGCTGTAAAGGAGCCAAA-3' (SEQ ID NO: 2)

15   2F(RRE6964E):

5'-ATCGGAATTCTTTTATTGTAAGATGGATTGGTTTTTAAAT-3' (SEQ ID NO: 3)

     2R(RRE-SA+BN):

5'-CGGGATCCGCGGCCGCGGATATGGATCTGTGGAGATAGAGGAACATAT-3' (SEQ ID NO:
4)

20   3F(SDFXhoSpe):

5'-TCGAGACTAGTGACTTGGTGAGTAGGCTT-3' (SEQ ID NO: 5)

     3R(SDR-Sal):

5'-TCGAAAGCCTACTCACCAAGTCACTACTC-3' (SEQ ID NO: 6)

     4F: 5'-AATTTCTCGAGCGGCCGCA-3' (SEQ ID NO: 7)

25   4R: 5'-AATTTGCGGCCGCTCGAGA-3' (SEQ ID NO: 8)

     5-1F(5LTRU3FKpn):

5'-GCGGTACCTGGATGGGATTTATTACTCCGATAGGA-3' (SEQ ID NO: 9)

     5-1R(GAGR-2Eco):

5'-GCGAATTCGATAGGGCTTGAAACATGGGTACTATTTCTGC-3' (SEQ ID NO: 10)

30   5-2F(RREF-EcoRI):

5'-GCGAATTCCCGTTTGTGCTAGGGTTCTTAGGCTTCT-3' (SEQ ID NO: 11)

     5-2R(RRESA+Rsac):

5'-TCCCCGCGGATATGGATCTGTGGAGATAGAGGAACATATC-3' (SEQ ID NO: 12)

     5-3F(3LTRF BS):

35   5'-GCGCGGCCGCGGATCCGTCGACGCACTTTTTAAAAGAAAAGGGA-3' (SEQ ID NO: 13)

     5-3R(3LTRR-SacI):

5'-GCGAGCTCTAATGCAGGCAAGTTTATTAGCTTTCTA-3' (SEQ ID NO: 14)

    6F(CMVFEcoSac):

5'-GGAATTCCCGCGGTAGTTATTAATAGTAATCAATTACGGG-3' (SEQ ID NO: 15)

    6R(EGFPRstoNB):

5   5'-CGGGATCCGCGGCCGCTTACTTGTACAGCTCGTCCATGCC-3' (SEQ ID NO: 16)

    9-1F(LTRF1 CMVU3F):

5'-TATATAAGCAGAGCTCGCTGGCTTGTAACTCAGTCTCTTA-3' (SEQ ID NO: 17)

    9-2F(LTRF2 EF1aU3F):

5'-TATATAAGTGCAGTACGCTGGCTTGTAACTCAGTCTCTTA-3' (SEQ ID NO: 18)

10   9-3F(CAGU3F):

5'-TATAAAAAGCGAAGCCGCTGGCTTGTAACTCAGTCTCTTA-3' (SEQ ID NO: 19)

   9R(GAGR-2Eco): 5'-GCGAATTCGATAGGGCTTGAAACATGGGTACTATTTCTGC-3'
(SEQ ID NO: 20)

    10-1F:

15   5'-CGGGGTACCTCAATATTGGCCATTAGCCATATTATTCATT-3' (SEQ ID NO: 21)

    10-1R(U3CMVR): 5'-AGTTACAAGCCAGCGAGCTCTGCTTATATAGACCTCCCAC-3'
(SEQ ID NO: 22)

    11F(LTRF LdU3FSalSC):

5'-ATGCGAGCTCGTCGACGCACTTTTTAAAAGAAAAGGGAGGACTGGATGGGATTTATTACTC

20 CGATAGGACGCTGGCTTGTAACTCAGTCTCTTACTAGG-3' (SEQ ID NO: 23)

    11R(3LTRR-SacI):

5'-GCGAGCTCTAATGCAGGCAAGTTTATTAGCTTTCTA-3' (SEQ ID NO: 24)

    <Preparation of DNA fragments by PCR>

    All PCR experiments were carried out using a PCR Supermix High
25 Fidelity (Gibco BRL). A pair of synthetic oligonucleotides as primers
were added at a final concentration of 1 nmol/ml to 90 μl of reaction
solution containing 1 μg of template DNA, and then the total volume
was adjusted to 100 μl with distilled water. The reaction was carried
out in a GeneAmp PCR System 9600 (Perkin Elmer). The PCR profile used
30 was as follows: pre-incubation at 94°C for 1 minute, 10 cycles of
denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds,
and extension at 68°C for 90 seconds, followed by incubation at 68°C
for 5 minutes. After reaction, samples were purified using Wizard
DNA Clean-up System (Promega), both ends of the PCR products were
35 treated with appropriate restriction enzymes, and fractionated in
a 1% low-melting temperature agarose gel (SeaPlaque GTG agarose, FMC

Biochem; dissolved in TAE buffer). DNA fragments of interest were excised from the gel and purified with Wizard PCR Preps DNA Purification System (Promega). The purified DNAs were used in ligation reaction.

<Construction of vectors>

5   Plasmids that provide backbone structures for vectors were constructed (Fig. 4). Namely, these plasmid were "packaging vector" providing proteins essential for the formation of vector particles in *trans*, "gene transfer vector" providing mRNA to be packaged into the vector and delivering and introducing genes of interest into target

10   cells, and the vector providing coat proteins participating in the formation of pseudotype vector particles.

Expression plasmids in each of which a sequence for gag, pol, tat, rev, vif, or vpr/x is located downstream of the promoter were constructed to provide proteins required for the formation of vector

15   particles. Most parts of the packaging signal Ψ and env were removed to avoid the generation of wild-type virus particles. An SD sequence was inserted upstream of gag and RRE sequence was inserted downstream of the first exon of tat/rev to allow the expression of all genes in the packaging vector. In addition, the entire nef sequence was

20   excluded because it was predicted to be nonessential for vector packaging (Fig. 4b).

The LTR sequences from the two ends of the genome, SD, Ψ, and RRE were inserted into the gene transfer vector providing RNA to be packaged into the vector. Further, a foreign promoter was substituted

25   for the region of 5' LTR promoter in the gene transfer vector. In addition, a self inactivating vector (SIN vector), in which a portion of the 3' LTR sequence was removed to prevent the transcription of full-length vector mRNA in target cells, was prepared; this vector contains as inserts the β-galactosidase gene as a reporter gene and

30   the CMV promoter for the expression of the reporter gene (Fig. 4c).

The vector used to provide VSV-G was the field-proven pVSV-G in pseudotyping retroviral and HIV vectors (Fig. 4d) (Burns, J. C. (1993) Proc. Natl. Acad. Sci. USA 90: 8033-8037).

More specific description is provided below.

35   <Construction of packaging vectors>

A DNA fragment corresponding to the region (5337 to 5770)

containing vif and the first exon of tat/rev was prepared by PCR using a pair of primers 1F and 1R and pSA212 as a template. A site for the restriction enzyme EcoRI had been added to one of the PCR primers, and thus the prepared DNA fragment contained an EcoRI site at its

5   3' end. The PCR fragment was digested with BglII and EcoRI, and purified by using agarose gel electrophoresis and Wizard PCR Preps DNA Purification System (Promega). The DNA fragment prepared as described above and another DNA fragment encoding the gag/pol region (from XhoI site (356) to BglII site (5338)) were ligated into

10   pBluescript KS+ (Stratagene) between the XhoI and EcoRI sites. Then, a Rev responsive element (RRE) and a DNA fragment corresponding to the region containing the second exon (6964 to 7993) of tat/rev were amplified by PCR. A NotI site was added to the 3' end by PCR using the pair of primers 2F and 2R and using pSA212 as a template by the

15   same procedure as used to prepare the above-described PCR fragment. The resulting PCR fragment was double-digested with EcoRI and NotI, followed by purification. The fragment was inserted into pBluescript KS+ containing gag-tat/rev between the EcoRI and NotI sites.

A DNA fragment (3F and 3R) comprising a sequence for splicing donor
20   (SD) site, which had synthesized to have an XhoI site and SalI site at 5' and 3' ends, respectively, was inserted at the XhoI site of the above-described pBluescript KS+ containing gag-RRE-tat/rev. The resulting plasmid was digested with XhoI and NotI. The fragment containing SD-gag-RRE-tat/rev was purified. A plasmid was prepared

25   by inserting an XhoI/NotI linker (4F and 4R) into pCAGGS (Gene, vol.108, pp.193-200, 1991) at the EcoRI site, and then the above-mentioned SD- gag-RRE-tat/rev fragment was inserted at the XhoI-NotI site. The plasmid obtained by the method as described above was used as the packaging vector pCAGGS/SIVagm gag-tat/rev.

30   <Construction of gene transfer vectors>

Using pSA212 as a template, PCR was carried out to amplify the SIVagm TYO1-derived 5' LTR region (8547 to 9053 + 1 to 982; containing KpnI site and EcoRI site at the 5' and 3' ends, respectively) using a pair of primers 5-1F and 5-1R; the 3' LTR region (8521 to 9170;

35   containing NotI and BamHI sites at the 5' end and SacI site at the 3' end) using a pair of primers 5-3F and 5-3R; and the RRE sequence

(7380 to 7993; containing EcoRI and SacII sites at the 5' and 3' ends, respectively) using a pair of primers 5-2F and 5-2R. The CMV promoter region (1 to 600; containing SacII and NotI sites at the 5' and 3' ends, respectively) derived from pEGFPN2 (Clontech) was amplified using a pair of primers 6F and 6R. The DNA fragments were digested at their ends. After purification, the 5' LTR, RRE, CMV promoter, and 3' LTR were inserted in this order into pBluescript KS+ at the KpnI-SacI site by ligation. A NotI fragment containing the β-galactosidase gene derived from pCMVβ (Clontech) was inserted as a reporter gene into the NotI site. The resulting plasmid was digested with KpnI and SacI to obtain a DNA fragment containing the region from the 5' LTR to the 3' LTR. The fragment was inserted into a control vector pGL3 at the KpnI-SacI site. The resulting plasmid was used as the gene transfer vector pGL3C/5' LTR.U3G2 /RREc/s/CMV F β-gal/WT 3' LTR.

Further, the CMV promoter region derived from pEGFPC2 (Clontech) and the region encoding EGFP (1 to 1330; containing a SacII site at the 5' end and NotI, BamHI sites, and translational stop codon at the 3' end) were amplified by PCR using a pair of primers 6F and 6R and using pEGFPC2 as a template. The four types of PCR fragments were digested with restriction enzymes KpnI and EcoRI, EcoRI and SacII, BamHI and SacI, and SacII and BamHI, respectively. After being purified, the fragments of 5' LTR, RRE, CMV promoter EGFP, and 3' LTR were inserted in this order into pBluescript KS+ between KpnI and SacI by ligation (pBS/5'LTR.U3G2/RREc/s/CMVFEGFP/WT3'LTR). The plasmid pBS/5'LTR.U3G2/RREc/s/CMVFEGFP/WT3'LTR was digested with KpnI and SacI to prepare a DNA fragment containing the region from the 5'LTR to the 3'LTR. The fragment was inserted into pGL3 (Promega) as a control vector at the KpnI-SacI site to construct a vector (pGL3C/5'LTR.U3G2/RREc/s/CMVFEGFP/WT3'LTR).

<Modification of 5' LTR>

A fragment containing gag region (9039 to 9170 + 1 to 982) downstream of the TATA box of 5' LTR was amplified by PCR using primers 9-1F, 2F, 3F and 9R and using pSA212 as a template. The CMV L promoter of cytomegalovirus (derived from pCI (Promega); nucleotides 1 to 721) was amplified by PCR using a pair of primers 10-1F and 10-1R. A fragment

containing a region downstream of the TATA box of 5' LTR was combined with the fragment containing the promoter. A DNA fragment containing a chimeric promoter of the promoter and the 5' LTR was prepared by PCR using the mixture as a template and a primer (10-1F) placed on
5   the 5' side of the promoter and another primer (9R) located on the 3' side of 5' LTR. The resulting DNA fragment was inserted into the gene transfer vector (pGL3C/5'LTR.U3G2/RREc/s/CMVFβ-gal/WT3'LTR) at the   KpnI-EcoRI   site   to   prepare pGL3C/CMVL.U3G2/RREc/s/CMVFβ-gal/WT3'LTR.   Similarly, the DNA
10 fragment obtained by the PCR experiment described above was also inserted into the vector pGL3C/5'LTR.U3G2/RREc/s/CMVFEGFP/WT3'LTR at   the   KpnI-EcoRI   site   to   prepare pGL3C/CMVL.U3G2/RREc/s/CMVFEGFP/WT3'LTR.

    <Preparation of 3' LTR-modified SIN vector (self inactivating
15 vector)>

    A DNA fragment containing the 27 bp at the 5' end, 15 bp at the 3' end, and R region from the U3 region of 3' LTR was amplified by PCR using a pair of primers 11F and 11R and using pSA212 as a template. This fragment was inserted into the SalI-SacI site of the gene transfer
20 vector pGL3C/CMVL.U3G2/RREc/s/CMVFβ-gal/WT3'LTR, which had been prepared to contain the chimeric promoter in the above Section, to prepare   pGL3C/CMVL.U3G2/RREc/s/CMVFβ-gal/3'LTRΔU3.   Similarly, this   fragment   was   also   inserted   into pGL3C/CMVL.U3G2/RREc/s/CMVFEGFP/WT3'LTR at the SalI-SacI site to
25 prepare pGL3C/CMVL.U3G2/RREc/s/CMVFEGFP/3LTRΔU3.

    <Preparation of constructed plasmid and confirmation of the structure>

    The plasmid was transformed into DH5α (Toyobo) by the conventional method, and incubated in agar plates. PCR was carried out using the
30 emerging colonies as templates and primers capable of recognizing the DNA sequences of the inserted DNA fragment and a portion corresponding to the insertion site of the plasmid. PCR was carried out using PLATINUM PCR Supermix (Gibco BRL). The presence and the size of amplified products were confirmed by agarose gel
35 electrophoresis to select clones containing desired inserts. Each clone was cultured in 10ml of LB culture medium, and then the plasmid

was purified with a QIAprep miniprep Kit (QIAGEN). The purified
plasmids were treated with restriction enzymes to digest both ends
of the inserted DNA fragments and electrophoresed in an agarose gel
to confirm the size of the DNA fragments. Clones where the plasmids
5   confirmed to contain an insert fragment of expected size were cultured
in 100 ml of LB culture medium. The plasmids were purified with a
QIAGEN Plasmid Maxi Kit (QIAGEN). Plasmids in which a PCR amplified
fragment had been inserted were purified from 3 or more clones and
sequenced, and the determined sequences were compared with those of
10   pSA212 to select clones containing no mutation.

    <Collection of vectors>

    293T cells were plated in a 6-well plastic culture plate at a cell
density of 5x 10^5 cells/well, and incubated under 10% CO_2 at 37°Cfor
48 hours. The culture medium was replaced with 800 µl/well of Opti
15   MEM before transfection. After 300 ng of the above-mentioned gene
transfer vector, 600 ng of the above-mentioned packaging vector, and
100 ng of the expression plasmid pVSV-G for VSV-G (Clontech) were
dissolved in 100 µl of Opti MEM in each well, 6 µl of PLUS Reagent
(Gibco BRL) was added thereto. The mixture was stirred, and then
20   allowed to stand still at room temperature for 15 minutes. 100 µl
of Opti MEM containing 4 µl of LIPOFECTAMINE reagent (Gibco BRL) was
added to the mixture. The resulting mixture was stirred and allowed
to stand still at room temperature for 15 minutes. The mixture was
added dropwise to the above-mentioned 293 T cells. The treated cells
25   were stirred gently and incubated under 10% CO_2 at 37°C for 3 hours.
1 ml of D-MEM containing 20% inactivated bovine serum was added to
each well, and the plate was incubated under 10% CO_2 at 37°C for 12
hours. After the culture medium was changed with 2 ml/well of D-MEM
containing 10% inactivated bovine serum, the plate was incubated for
30   24 hours. Then, the culture supernatant was collected, and filtered
with a filter with 0.45-µm diameter pores. The filtered supernatant
was used in the subsequent experiments.

    <SIVagm vector-mediated gene transfer>

    293T cells were plated in a 6-well plastic culture plate at a cell
35   density of 5x 10^5 cells/well, and incubated under 10% CO_2 at 37°Cfor
48 hours. The culture medium was removed, and 1 ml of the solution

containing the vector solution to which polybrane (Sigma) had been added at a final concentration of 8 μg/ml was overlaid onto the cells. The cells were incubated under 10% CO_2 at 37°C for 3 hours to achieve vector transfect. After three hours, 1 ml of the culture medium was

5 added to the cells. On the following day, the culture medium was changed. Next day, when the vector used was the β-Gal expression vector, staining was carried out using X-gal as the substrate with a β-Gal Staining Kit (Invitrogen), and the expression of β-galactosidase in the target cells was observed under a light

10 microscope. However, when the vector used was the EGFP expression vector, the expression was analyzed under a fluorescence microscope.
    <Vector titration>
    Vector titration was carried out by calculating the number of cells into which a gene was introduced using 1 ml of the vector solution.

15 293T cells were plated in a 6-well plastic culture plate at a cell density of 1x 10^6 cells/plate, and incubated for 48 hours. By the same procedure as described above, infection to the cells was carried out with 1 ml of the vector solution. X-gal staining was performed 48 hours after infection. The mean value for the number of cells

20 containing the transferred gene in a visual field was determined from three different visual fields under a light microscope with 200-fold magnification, and multiplied by the coefficient 854.865 that had been determined based on the area of the visual field and the area of the plate to determine the titer. The unit of titer was defined

25 as Transducing Unit (T.U.)/ml. In addition, p27 protein in the vector solution was quantified using an SIV core antigen EIA kit (Coulter).
    <Evaluation of vectors>
    It was predicted that the enhancement of the activity of 5' promoter in the gene transfer vector resulted in increased levels of RNA

30 transcription and, which improved packaging efficiency and therefore increased vector titer. Thus, vectors where the 5' promoter had been replaced were evaluated based on the efficiency of gene transfer into 293T cells. The gene transfer was carried out by the same procedure as described above, and the expression of β-galactosidase was

35 visualized by X-gal staining, which was followed by observation under a light microscope.

An SIN SIV vector was prepared based on an SIN gene transfer vector whose 3' LTR promoter had been deleted. An SIV vector was also prepared based on a gene transfer vector containing the original wild-type 3' LTR. The efficiency of transfer into 293T cells was compared between

5 the two types of vectors. The amount of p27 protein was also determined to compare the transfer efficiencies normalized with the protein amount. In addition, the achievement of EGFP gene transfer into cells-cycle arrested 293T cells by irradiation, and SH-SY5Y cells terminally differentiated by retinoic acid were confirmed by

10 observations under a fluorescence microscope.

<Results obtained by gene transfer into 293T cells>

The β-galactosidase gene was introduced into human fetal kidney-derived 293T cells using the SIVagm vector. The cells containing the transferred gene were stained using X-gal as the

15 substrate. As Fig. 5 shows, cells stained blue were detected, which indicates that the β-galactosidase gene was expressed in the cells. According to the experimental result, the efficiency of gene transfer was 0.5 to 1x 10^6 T.U. with 1 ml of vector solution. The amount of p27 in the vector solution was 0.5 to 1 μg/ml, and the transfer

20 efficiency per 100 ng of p27 was 10^5 T.U.

<Assessment for the performance of SIN vector>

The transfer efficiency of the SIN vector was compared with that of a conventional vector containing wild-type 3' LTR prepared under the same conditions. While the titer of conventional vector was 2.4

25 to 2.8 T.U./ml, that of SIN vector was 2.5 to 2.9 T.U./ml; thus, the transfer efficiency of SIN vector was 105% when the efficiency of conventional vector was taken as 100%. Further, the relationship between the transfer efficiency and the amount of p27 protein was determined by measuring the efficiency of transfer into 293T cells

30 and assaying the amount of p27 by EIA; the efficiency of SIN vector was 7x 10^5 T.U. per 100 ng of p27.

The EGFP gene was transferred by the SIN vector into cells-cycle arrested 293T cells and terminally differentiated SH-SY5Y cells. 293T cells were cultured in DMEM containing 10% FCS under 10% CO_2 at

35 37°C. The cells cycle was arrested in G1-S phase by adding aphidicolin (Calbiochem-Novabiochem International, Inc.) at a final

concentration of 20 µg/ml (Huberman, J.A. et al., Cell: Vol.23, 647-648. 1981; Byrnes, J.J., Mol. Cell. Biochem.: Apr, 62(1), :13-24. 1984). G2-M phase arrest was achieved by irradiating 4000-rad X ray to 2×10^7 cells (Kastan, M.B. et al., Cell: Nov 13, 71(4):587-97. 1992).

5   24 hours after the treatment for cell arrest, the cells were plated in a 6-well culture plate (SUMILON) coated with collagen type I at a cell density of 1×10^6/well. After 24 hours, gene transfer was carried out. Alternatively, human neuroblast cell line SH-SY5Y (Koshizawa, S.H. et al., Cancer Lett.: Jan 1, 111(1-2), 117-25. 1997) was cultured

10   in RPMI1640 containing 10% inactivated calf serum under 5% CO_2 at 37°C, and all-trans retinoic acid (SIGMA) was added to the culture medium at a final concentration of 5 µM. Cells were incubated for 7 days, and then gene transfer was carried out (Odelstad, L. et al., Brain Res.: Nov 9, 224(1), 69-82. 1981).

15   The top panel of Fig. 6 shows a pattern of EGFP expression in 293T cells observed with fluorescence microscopy. High efficiency expression of the gene was recognized. The bottom panel of Fig. 6 shows EGFP expression in SH-SY5Y. EGFP expression was found in cells having nurites which appeared to have differentiated into neurons.

20   The SIVagm vector prepared in this Example was confirmed to mediate gene transfer into culture cells highly efficiently. The probability of reconstitution of wild-type virus particles in vector packaging after co-transfection of the three types of independent plasmids is assumed to be very low. Furthermore, SIVagm TYO-1 itself, used as

25   a base for the vector, has been confirmed to exhibit no pathogenicity in terms of both natural and experiment infection (Ohta, Y. et al., Int. J. Cancer: 41, 115-22, 1988; Miura, T. et al., J. Med. Primatol.: 18(3-4), 255-9. 1989; Honjo, S. et al., J. Med. Primatol. 19(1), 9-20, 1990). In addition, the vector can be highly safe because generally

30   lentiviruses are highly species-specific and have only weak pathogenicity to animal species other than their original target species (Novembre, F. J. et al., J. Virol.: 71(5), 4086-91. 1997).

In this Example, the packaging signal sequence has been removed from the packaging vector construct, and thus the RNA encoding viral

35   proteins is not packaged into particles. The binding of rev protein to RRE induces transfer of RNA into the cytoplasm and suppression

of splicing, resulting in the expression of viral proteins and packaging of full-length RNA into virus particles. Therefore, the inserted RRE in both packaging vector and gene transfer vector can regulate mRNA splicing of the packaging vector, which thus can allow

5   expression of all genes. Then, mRNA of the gene transfer vector can be transferred into the cytoplasm, and packaged into the vector particles. There are some cases where vif, vpr/x have been excluded from HIV-1 vectors (Dull, T. et al., J. Virol.: Nov, 72(11), 8463-71. 1998), and this suggests the possibility that proteins are not

10 essential for packaging and functioning of vector particles. vpr has been believed to be a factor responsible for the infectivity to nondividing cells (Heinzinger, N. K. et al., Proc. Natl. Acad. Sci. U S A: Jul 19, 91(15), 7311-5, 1994); a report describes that the type of cell, to which HIV-1 vector can transfer genes, varies depending

15 on the presence of vpr (Kim, V. N. et al., J. Virol.: Jan, 72(1), 811-6. 1998). It has also been reported that nef, which was completely excluded form the packaging vector in the Examples described herein, can be a causative protein of the SIV-mediated immunodeficiency based on the evidence obtained by experiments with infection to monkey (von

20 Gegerfelt, A. S. et al., J. Virol. 73, 6159-65, 1999; Kestler, H. W. 3d., Naidu, Y. N., Kodama, T., King, N. W., Daniel, M. D., Li, Y., Desrosiers, R. C. Use of infectious molecular clones of simian immunodeficiency virus for pathogenesis studies., J. Med. Primatol. 18(3-4): 305-9, 1989). The corresponding sequence has been removed

25 completely from the SIVagm vector constructed in this Example; therefore, even if reconstituted virus particles containing viral genes derived from the packaging vector were formed, the risk of pathogenicity for such particles would be further decreased.

The lentivirus-based vector can transfer genes into cell-cycle

30 arrested culture cells and neurons because the original lentivirus is infectious to nondividing cells (Naldini, L. et al., Science: 272 263-267, 1996; Sutton, R. E. et al., J. Virol., 73(5), 3649-60, 1999). When such a vector has been pseudotyped by VSV-G, unlike the original SIV, the infectivity of the vector is not limited to the infection

35 to CD4- and chemokine receptor-positive cells. The receptor of VSV-G is known to be phosphatidylserine, which is one of the phospholipids,

whose molecules are present on the surface of various types of cells (Schlegel, R. et al., Cell, 32(2), 639-46, 1983). Thus, the SIVagm vector which has been pseudotyped by VSV-G has a considerably wider range of infectivity. It is predicted that when viruses that are pseudotyped by membrane proteins having hemagglutinin activity are prepared based on this vector, they are capable of transferring genes to almost all types of animal cells with a high degree of efficiency.

[Example 6] Preparation of virosome

The seed of wild-type SeV (Z strain) was inoculated to fertile eggs (10-day eggs), and the eggs were cultured at 35.3°C for three days. Then, chorio-allantoic fluid was collected from the eggs. After centrifugation at 4,000 rpm for 15 minutes, the supernatant was centrifuged at 10,000 rpm for one hour to precipitate virus particles. The pellet was resuspended in BSS, and overlaid on a solution with a sucrose density gradient (30%/50%), followed by centrifugation at 25,000 rpm for one hour. A band of virus particles formed at the boundary between 30% and 50% sucrose was collected, and then diluted with a large volume of BSS. Then, the sample was centrifuged at 9,000 rpm for one hour to precipitate virus particles. The resulting virus particles were resuspended in BSS and stored at -80°C until used.

F-HN virosome and F virosome were prepared from the Sendai virus obtained according to the method as described in the reference (Bagai et al., 1993, Biochem. Biophys. Acta 1152, 15-25). Specifically, the Sendai virus was solubilized with a detergent, and then insoluble RNP was removed by centrifugation. The particles were reconstituted by removing the detergent from the solution where F and HN proteins, and lipids (envelope lipids) had been solubilized to prepare F-HN virosome. The preparation was carried out under the same conditions as used in Example 7 except that the concentration of Triton X-100 was 1% (v/v) before centrifugation. The Sendai virus was pretreated with DTT (dithiothreitol) to reduce HN protein, and solubilized with a detergent by the same procedure as described above. The resulting insoluble HN protein along with RNP was removed by centrifugation. The particles were reconstituted to prepare F virosome by removing

the detergent from the solution by the same procedure as described above.

The resulting virosomes exhibited heterogeneous particle diameters. Therefore, the virosomes were separated into two fractions containing the particles of different size through centrifugation at 12,000 rpm for 10 minutes (small virosome: average particle diameter = 140 nm; large virosome: average particle diameter = 1.4 μm). The fractionated virosomes were assessed by SDS-PAGE. Both F protein and HN protein were detected in the F-HN virosome; F protein, in the F virosome. Thus, it was confirmed that the virosomes nearly desired had been obtained (Fig. 7).

The virosome prepared as described above or Sendai virus inactivated by UV irradiation was fused with EGFP-expressing SIV virus, which had been prepared and enriched by the method described in Example 7(3) under conditions at 37°C for two hours with the composition indicated in the Table below. It has been reported that the efficiency of fusion between VSV-G and liposome is higher at weakly acidic pHs (Yamada, S. et al., 1986, Biochemistry, 25, 3703-3708). Therefore, the fusion was carried out at pH5.5 or neutral pH.

Table 3

	SIV			SeV		
Condition	Titer (TU/ml)	Buffer	Volume (μl)	Virus	Buffer	Volume (μl)
1	1×10^8	BSS	50	UV inactivated SeV 1×10^9 pfu/ml	BSS	50
2	1×10^8	pH5.5 Citric acid	50	UV inactivated SeV 1×10^9 pfu/ml	pH5.5 Citric acid	50
3	1×10^8	BSS	50	FHN virosome (small)	BSS	50
4	1×10^8	BSS	50	FHN virosome (large)	BSS	50
5	1×10^8	BSS	50	F virosome (small)	BSS	50
6	1×10^8	BSS	50	F virosome (large)	BSS	50

The mixture prepared as described above was added to the culture supernatant of HeLa cell at MOI=10. A control experiment was carried

5   out, where SIV was simply co-infected with Sendai virus. In this experiment, first, 5 minutes after SIV was added to culture medium, Sendai virus was added in 10-times volume of SIV. After the viruses were added to the culture medium, the mixture was incubated for infection for 10, 30, or 180 minutes. Then, the culture medium was

10   changed with fresh one. 48 hours after the start of infection, observation was made to detect GFP fluorescence (Fig. 8).

According to the result obtained, when SIV alone infected HeLa cells and the duration of contact was 30 minutes or shorter, the efficiency of infection was very low. It was found that the mixture

15   of fusion of SIV and UV-inactivated Sendai virus had a tendency to infect faster than SIV alone. The infection efficiency at neutral pH was higher than that at pH 5.5. This may result from decreased influence of pH on SIV at a neutral.

The fusion with F-HN virosome also enhanced infection, thereby

20   significantly improving infection efficiency. F virosome also improved the infection efficiently but to a lesser extent as compared with that of F-HN virosome. When SIV was simply co-infected with Sendai virus, the infection efficiency was only comparable to that of SIV alone.

25   These results demonstrate that fusion of SIV with Sendai virus

or F-HN virosome enhances infectivity of SIV. Furthermore, it was suggested that HN protein of Sendai virus largely contributed to the enhancement of infection efficiency.

5 [Example 7] Preparation and evaluation of SIV-SeV fusion vector

(1) Preparation of wild-type SeV

The seed of wild-type SeV (Z strain) was inoculated to fertile eggs (10-day eggs), and the eggs were cultured at 35.3°C for three days. Then, chorio-allantoic fluid was collected from the eggs.
10 After centrifugation at 4,000 rpm for 15 minutes, the supernatant was centrifuged at 10,000 rpm for one hour to precipitate virus particles. The pellet was resuspended in BSS, and overlaid on a solution with a sucrose density gradient (30%/50%), followed by centrifugation at 25,000 rpm for one hour. A band of virus particles
15 formed at the boundary between 30% and 50% sucrose was collected, and then diluted with a large volume of BSS. Then, the sample was centrifuged at 9,000 rpm for one hour to precipitate virus particles. The resulting virus particles were resuspended in BSS and stored at −80°C for later use.

20 (2) Reconstitution of virosome from SeV transmembrane protein

1. FHN virosome

0.2 ml of 20% (V/V) Triton X-100/BSS was added to 1.8 ml of SeV ($OD_{540}=5$, BSS), and the mixture was allowed to stand still at room temperature for 1 hour to solubilize SeV. In Example 6, the virosomes
25 exhibited heterogeneous particle diameters. Thus, the concentration of Triton X-100 was adjusted to 2%(v/v) before centrifugation. The insoluble RNP was precipitated by centrifugation (at 100,000x g at 4°C for 1 hour), and then the supernatant where F and HN proteins had been solubilized was collected. Bio-bead SM-2 (BIORAD) was added
30 to the supernatant in three steps (0.5g x twice, at room temperature for 1 hour; 1g x once, at 4°C for 15 hours plus incubation at room temperature 1 hour) to remove the detergent by adsorption, followed by the removal of the beads. Thus, approximately 1.5 ml of FHN virosome was obtained.

35 2. F virosome

0.2 ml of 30 mM dithiothreitol/BSS was added to 2 ml of SeV ($OD_{540}=5$,

BSS), and the mixture was incubated at 37°C for two hours to irreversibly reduce HN protein. After dilution with BSS, virus particles were separated by centrifugation, and resuspended in 1.8 ml of BSS. 0.2 ml of 20% (V/V) Triton X-100/BSS was added to the virus

5   suspension, and the mixture was allowed to stand still at room temperature for one hour to solubilize SeV. Then, insoluble RNP and reduced HN protein were removed by the same procedure as described above. The supernatant in which F protein had been solubilized was collected. Approximately 1.5 ml of F virosome was obtained by the

10 same procedure as used to prepare FHN virosome.

    3. HN virosome

    2 ml of 150 units/ml trypsin (Sigma)/BSS was added to 2 ml of SeV $(OD_{540}=5, BSS)$, and the mixture was incubated at 37°C for two hours to digest F protein. Then, 1 mg/ml trypsin-chymotrypsin inhibitor

15 (Sigma)/BSS was added to the reaction mixture to stop the reaction. After dilution with BSS, virus particles were separated by centrifugation, and resuspended in 1.8 ml of BSS. Approximately 1.5 ml of HN virosome where the function of F protein had been inactivated was obtained by the same procedure as used to prepare FHN virosome.

20 The characteristics of the three types of virosomes are shown in the Table below. The data of HA activity representing the function of F and HN proteins, hemolytic activity, and electrophoretic patterns showed that virosomes were required (Fig. 9).

Table 4

	FHN Virosome	F Virosome	HN Virosome
HA	+	–	+
Hemolytic Activity	+	–	–
OD_{540}	0.480	0.491	0.470
Protein Concentration (mg/mL)	0.96	0.57	0.78
Average Particle Diameter (nm)	143.5	168.9	133.0

    (3) Preparation of SIV-LacZ

    SIV containing LacZ gene (SIV-LacZ) was prepared using the

35 following three types of plasmids.

        □Packaging vector: pCAGGS/SIVagm gag-tat/rev

□Gene transfer vector :

  pGL3C/CMVL.U3G2/RREc/s/CMVFEGFP/3LTRΔU3   or

  pGL3C/CMVL.U3G2/RREc/s/CMVF β-gal/3LTRΔU3

□VSV-G plasmid: pVSV-G

5   150 µg of packaging vector, 300 µg of gene transfer vector, and 50 µg of VSV-G plasmid were dissolved in 75 ml of DMEM. 2 ml of Plus reagent (Life Technologies Oriental, Inc.) was added to the solution, and the resulting mixture was allowed to stand still at room temperature for 15 minutes. A suspension prepared by combining 3 ml of 10 LipofectAMINE reagent (Life Technologies Oriental, Inc.) and 75 ml of DMEM was added to the mixture, and the resulting mixture was allowed to stand still at room temperature for 15 minutes to form DNA complex. 3 ml of the DNA complex solution was added dropwise to 293T cells (which were prepared in 50 dishes containing 10% FCS/DMEM at a cell 15 density of 2.5×10^6 cells/150-mm dish, 2 days before transfection). Transfection was carried out under 10% CO_2 at 37°C for 3 hours. 10 ml of 10% FCS/DMEM was added to the cells, and then the incubation was further continued. After 48 hours, the culture supernatant was collected and filtered with a membrane filter with 0.45-µm diameter 20 pores. The filtrate was centrifuged at 42,500x g for 1.5 hours to precipitate virus particles. The precipitates were combined together and resuspended in 15 ml of reverse transcription solution (100 µM dNTPs, 3mM Spermine, 0.3 mM Spermidine, 10 mM $MgCl_2$/TBS). Revers transcription was carried out at 37°C for two hours. Then, the virus 25 was centrifuged (at 42,500x g for two hours), and the particles were resuspended in 500 µL of PBS (containing 5% FCS and 2 µg/mL polybrene) to obtain an SIV-LacZ-enriched solution. The titer of SIV-LacZ was approximately 5×10^8 T.U./mL (293T cells).

    (4) Preparation of fusion vector

30   SIV-LacZ was diluted to 1×10^8 T.U./mL with BSS. An equal volume of a virosome/BSS solution was added to this solution. The mixture was allowed to stand still at 4°C for 30 minutes for adsorption. Then, the fusion was carried out at 37°C for two hours, and the reaction solution was diluted 10 times with ice-cold PBS (at a final SIV 35 concentration of 5×10^6 T.U./mL). This SIV was used immediately in infection experiments.

(5) Infection experiments

1 ml of 293T cells in 10% FCS/DMEM (hereinafter referred to as culture medium) was added to each well of 12-well plates at a cell density of 1x 10^5 cells/well, and the cells were cultured under 10%
5 CO_2 at 37°C for 48 hours. Immediately before infection, the culture medium was reduced to 0.5 ml. A fusion vector consisting of SIV and the virosome was added to each well at a concentration of 5x 10^6 T.U./ml (100 µl/well), and the mixture was incubated under 10% CO_2 at 37°C for 10 minutes for infection. After the wells were washed twice with
10 culture medium, 2 ml of culture medium was added to each well. Then, the plates were further incubated. After 48 hours, the cells were washed twice with PBS, and lysed with 0.25 ml of cell lysis buffer (PicaGene LC-β; Toyo Ink). The cell lyates were centrifuged at 12,000 rpm for 3 minutes, and the supernatant was diluted appropriately.
15 The expression level of the LacZ gene was determined using Galacto-Light Plus™ (TROPIX, Inc.). The data was presented in relative LacZ activity per 1 µg of protein from the cells (RLU/µg protein), which was obtained as a mean ± standard error from measurements in triplicate.
20 The expression level of LacZ was significantly greater with the FHN-virosome fusion than that with SIV alone (p<0.05). Unlike FHN-virosome, F-virosome did not result in increased expression levels of the gene. HN-virosome also had a tendency to elevate the level but to a lesser extent than FHN (Fig. 10).
25 In the above vectors, SIV VSV-G protein and virosome F protein are responsible for the fusion. The optimal pH for the fusion using VSV-G protein falls within a range of weakly acidic pH. On the other hand, F protein has been reported to participate in the fusion even at a neutral pH. In this Example, the fusion was achieved at a neutral
30 pH, and thus F protein of SeV was assumed to be responsible for the majority of the fusion. It has been reported that fusion between SeV and liposome is achievable, as long as F protein is functionally active. Thus, when the efficiency of FHN virosome-based fusion is hypothesized to be comparable to that of the F virosome-based fusion, the increased
35 level of gene expression by FHN virosome alone can be attributed to the substantial contribution of the additional HN. HN virosome also

exhibited an effect at certain level; this suggests that VSV-G-based fusion occurred to some extent, which resulted in the integration of HN protein into SIV.

On the other hand, the effect of simple co-infection of SIV and virosome was comparable to that of SIV alone (Fig. 10). These findings suggest that the fusion was established through incubation at 37°C of the mixture containing the two, and thus the membrane proteins from SeV were integrated into the SIV envelope; HN protein mainly contributed to the improvement of the infection rate.

[Example 8] Preparation and performance analysis of HN pseudotype lentivirus vector

1. Cell culture

293T cells (human fetal kidney cell line) were cultured in Dulbecco's Modified Eagle Medium (DMEM)-High glucose (Gibco BRL) containing 10% inactivated calf serum (BIO WHITTAKER) under 10% CO_2 at 37°C.

2. Preparation of vector

293T cells were plated in a 6-well plastic culture plate at a cell density of 5x 10^5 cells/well, and then incubated under 10% CO_2 at 37°C for 48 hours. The culture medium was changed with 800 μl/well of DMEM containing 1% bovine serum albumin. The cells were then used in transfection. In the combinations as indicated in Table 5 below, 1200 ng of gene transfer vector (pGL3C/CMVL.U3G2/RREc/s/CMVFEGFP/3LTRΔU3 or pGL3C/CMVL.U3G2/RREc/s/CMVF β-gal/3LTRΔU3), 360 ng of packaging vector (pCAGGS/SIVagm gag-tat/rev), 120 ng of VSV-G expression plasmid pVSV-G(Clontech), 240 ng each of Sendai virus HN, F, and M protein expression plasmids, pCAGGS-HN, pCAGGS-F, and pCAGGS-M, were dissolved in 100 μl of Opti MEM in each well. Then, 6 μl of PLUS Reagent (Gibco BRL) was added to the well. The mixture was stirred, and then allowed to stand still at room temperature for 15 minutes. A solution obtained by diluting 4 μl of LIPOFECTAMINE Reagent (Gibco BRL) with 100 μl of Opti MEM was added to the mixture. The mixed solution was stirred, and then allowed to stand still at room temperature for another 15 minutes. The resulting mixture was added dropwise to the 293 T cells prepared above while being stirred gently. The cells were then

incubated under 10% CO_2 at 37°C for 3 hours. 1 ml of DMEM containing 1% bovine serum albumin and 15 μg/ml trypsin (Gibco BRL) was added to each well. After culturing under 10% CO_2 at 37°C for 48 hour, the culture supernatant was collected and filtered with a filter with 0.45-μm diameter and the resulting solution was used as a vector solution.

Table 5

	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩	⑪	⑫
PV	■	■	■	■	■	■	■	■	■	■	■	
GTV	■	■	■	■	■	■	■	■	■	■	■	
VSV-G	■					■	■	■	■	■	■	
HN		■		■		■	■		■		■	
F			■		■		■	■		■	■	
M				■		■		■		■	■	

(In this Table, plasmids added are indicated in black. "PV" indicates packaging vector pCAGGS/SIVagm gag-tat/rev; "GTV", gene transfer vector pGL3C/CMVL.U3G2/RREc/s/CMVFEGFP/3LTRΔU3 or pGL3C/CMVL.U3G2/RREc/s/CMVFβ-gal/3LTRΔU3; "VSV-G", VSV-G expression plasmid pVSV-G; "HN", "F", and "M" indicate the Sendai virus HN protein-, F protein-, and M protein-expressing plasmids, pCAGGS-HN, pCAGGS-F, and pCAGGS-M, respectively.)

3. Large-scale preparation and enrichment of vector

293T cells were plated in 15-cm plastic dishes at a cell density of 5x 10^6 cells/dish, and cultured under 10% CO_2 at 37°C for 48 hours. The culture medium was changed with 10 ml/dish of DMEM containing 1% bovine serum albumin. The cells were then used in transfection. In the combinations as indicated in Table 5 above, 8 μg of gene transfer vector, 2.4 μg of packaging vector, 0.8 μg of VSV-G expression plasmid pVSV-G (Clontech), 1.6 μg each of Sendai virus HN and F protein expression plasmids, pCAGGS-HN, pCAGGS-F, and pCAGGS-M, were dissolved in 1.5 ml of Opti MEM in each dish. Then, 40 μl of PLUS Reagent (Gibco BRL) was added to each dish. The mixture was stirred, and then allowed to stand still at room temperature for 15 minutes. A solution obtained by diluting 60 μl of LIPOFECTAMINE Reagent (Gibco

BRL) with 1.5 ml of Opti MEM was added to the mixture. The mixed solution was stirred, and then allowed to stand still at room temperature for another 15 minutes. The mixture was added dropwise to the 293 T cells prepared above while being stirred gently. The cells were then
5   incubated under 10% CO_2 at 37°C for 3 hours. 10ml of DMEM containing 1% bovine serum albumin and 15 µg/ml trypsin (Gibco BRL) was added to each well. After culture under 10% CO_2 at 37°C for 48 hour, the culture supernatant was collected, and filtered with a filter with 0.45-µm diameter pores, followed by centrifugation at 42,490x g (TOMY
10   SRX-201, TA21BH) at 4°C for 90 minutes. The resulting pellet was dissolved in a 1/100 volume of a reverse transcription solution (TBS, 10 mM $MgCl_2$, 3 mM SPERMINE, 0.3 nM SUPERMIDINE, 100 mM dNTPs), and the solution was incubated at 37°C for two hours. After reverse transcription reaction, the reaction was centrifuged at 42,490x g
15   (TOMY SRX-201, TA21BH) at 4°C for two hours, and the resulting pellet was dissolved in PBS (5% FCS, 2 µg/ml polybrene). The solution was stored at -80°C until used.

    When the three types of plasmids of packaging vector, gene transfer vector, and coat protein expression vector are co-transfected into
20   cells, mRNA is transcribed from the gene transfer vector; the recognition of the Ψ sequence by a viral protein provided by the packaging vector allowed the RNA packaging into vector particles. Then, vector particles are finally formed via pseudotyping with the envelope protein provided by the coat protein vector. The prepared
25   pseudotype virus vector was tested for the ability to achieve gene transfer into cells.

    <Vector titration and detection of cells containing transferred gene>

    Vector-mediated gene transfer into 293T cells was carried out by
30   the above-mentioned method. The titer was determined based on the number of cells into which genes had been introduced using 1 ml of the vector solution. When a gene transfer vector encoding EGFP was used as a reporter gene, target cells were fixed with PBS (Invitrogen) containing 2% formaldehyde and 0.2% glutaraldehyde at room temperature
35   for 20 minutes, and washed once with PBS; EGFP expression was evaluated based observations under a fluorescence invert microscope

(DMIRB(SLR), Leica).

The three types of plasmids of packaging vector (pCAGGS/SIVagm gag-tat/rev), gene transfer vector (pGL3C/CMVL.U3G2/RREc/s/CMVFEGFP/3LTRΔU3), and Sendai virus-derived coat protein vector (pCAGGS-F, pCAGGS-HN, and pCAGGS-M) were co-transfected into cells in the combinations indicated in (2) to (5) of Table 5. In any of these combinations, the gene was not transferred into 293T cells. However, gene transfer was successfully achieved by the co-expression with VSV-G. These findings demonstrated that, when prepared with F and HN proteins alone, the pseudotype SIVagm vector did not mediate sufficiently high efficiency of gene transfer, but pseudotype viral vectors exhibiting higher infectivity could be produced by the help of coexisting viral envelope proteins, such as VSV-G, having infectivity to human cells.

The EGFP gene was introduced into 293T cells using the pseudotype SIVagm vector, and the vector titer was determined; the titer of prepared viral vector was 0.5 to 1x 10^5 T.U. per 1 ml of the vector solution. The titer of viral vector enriched by centrifugation was as high as 1.0x 10^7 T.U./ml. This demonstrated that the SIVagm vector pseudotyped by Sendai virus F and HN proteins could be enriched by centrifugation.

[Example 9] *In vivo* administration of pseudotype viral vector

The mucosal cells of tracheal epithelium into which genes are difficult to introduce with a conventional viral vector should be pre-treated to remove physical barriers at the time of introduction. Thus, when one intends to introduce genes into such cells by the method using the VSV-G pseudotype HIV vector, sufficient effects are ensured only after the cells are damaged with sulfur dioxide, etc. (L. G. Johnson et al., Gene Therapy: 7, 568-574. 2000). Thus, it was tested whether the SIVagm vector pseudotyped by Sendai virus F and HN proteins could transfer genes with high efficiency into mucosal cells of tracheal epithelium without damaging the cells.

6-week old C57BL/6 mice (male) were anesthetized by seboflurene inhalation, and the EGFP-expressing FHN pseudotype SIV vector (EGFP expression vector in (11) of Table 5) (referred to as SIV-F/HN/M-EGFP),

which had been produced by the procedure described above, was administered to them intranasally. The tissues were embedded in OCT compound and sliced into frozen sections; the sections were observed under a fluorescence microscope (Zeiss).

5   100 µl of SIV-F/HN/M-EGFP (10^8 T.U.) was administered intranasally, the trachea was observed after 3 days. EGFP fluorescence was detected in epithelial cells of the trachea (Fig. 11). In the same individuals, the mucosal epithelium also confirmed to express the gene, and the fluorescence was found to be localized

10   in the pseudostratified ciliated epithelium in septonasal mucus membrane (Fig. 12).

[Example 10] Persistent expression of foreign genes mediated by *in vivo* administration of pseudotype virus vector

15   A pseudotype retrovirus containing F and HN proteins was produced using a gene transfer vector expressing EGFP by the same procedure as described above. Specifically, pseudotype MSCV (MSCV-F/HN/M-EGFP) corresponding to (9) of Table 2 and pseudotype SIV (SIV-F/HN/M-EGFP) corresponding to (11) of Table 5 were produced.

20   VSV-G pseudotype SIV (SIV-VSV-EGFP) corresponding to (1) of Table 5 was used as a control. The EGFP expression viral vectors were administered to mice intranasally, and sections from nasal mucous membrane tissues were prepared 90 days after the administration to observe EGFP expression.

25   According to the results obtained, every vector used resulted in EGFP expression; intense fluorescence signals for EGFP were detected in the cases of administration of MSCV-F/HN/M-EGFP and SIV-F/HN/M-EGFP containing F and HN proteins. In particular, administration of SIV-F/HN/M-EGFP vector gave a more intense fluorescent signal than

30   that observed with the other vectors. Thus, the vector was confirmed to have the ability to transfer genes with high efficiency (Fig. 13).

[Example 11] Construction of novel expression plasmid for Sendai virus envelope proteins

35   [1] Construction of expression plasmid for cytoplasmic domain-substituted HN protein

An HN expression plasmid was constructed, where the cytoplasmic domain of HN protein was replaced with the cytoplasmic domain of SIV envelope protein (Fig. 14). Three pairs of synthetic oligonucleotides (Xho+Xma/Xma-Xho, Xma+131/135-Xma, 132+Bam/Bam-136) were annealed, and then each was inserted into pBluescript KS+ (Stratagene) at the XhoI-BamHI site. A purified fragment containing the synthetic oligonucleotide, which had been obtained by digesting the above-described recombinant plasmid with XhoI and DraIII, or a purified fragment containing a 3' portion of HN protein, which had been obtained by digesting the HN protein expression plasmid pCAGGS-HN with DraIII and Bsu36I, was inserted into pCAGGS (Gene, vol.108, pp.193-200, 1991) at the XhoI-Bsu36I site. The plasmid obtained by the method described above was used as the expression plasmid for SIV cytoplasmic domain-substituted HN protein, pCAGGS-SIVct/HN.

[2] Construction of expression plasmid for SIV cytoplasmic domain-added HN protein

An HN expression plasmid was constructed, where the cytoplasmic domain of SIV envelope protein was added to HN protein (Fig. 15). A region containing the cytoplasmic domain of SIV envelope protein and a portion of HN protein was amplified by PCR using primers FSIVhn and RhnSIV, and using as a template the above-mentioned expression plasmid for cytoplasmic domain-substituted HN protein. After the amplified fragment was digested with XhoI and AccI, the three pairs of synthetic oligonucleotides prepared in the above [1] were inserted into pBluescript KS+ (Stratagene) at the XhoI-AccI site to replace with the fragment containing the cytoplasmic domain of SIV envelope.

A purified fragment containing the synthetic oligonucleotide, which had been obtained by digesting the recombinant plasmid with XhoI and DraIII, or a fragment containing a 3' portion of HN protein, which had been obtained by digesting the HN protein expression plasmid pCAGGS-HN with DraIII and Bsu36I, was inserted into pCAGGS (Gene, vol.108, pp.193-200, 1991) at the XhoI-Bsu36I site. The plasmid obtained by the method described above was used as the expression plasmid for SIV cytoplasmic domain-added HN protein, pCAGGS-SIVct+HN.

[3] Construction of F protein expression plasmid lacking the cytoplasmic domain

F protein expression plasmids were constructed, each of which contained the first 27, 14, or 4 residues from the 5' end of the cytoplasmic domain amino acids of F protein and thus lacked 15, 28, or 38 amino acid residues, respectively (Fig. 16). Each of the fragments lacking 15, 28, and 38 amino acids, respectively, was amplified by PCR using the pairs of primers, XhFF and NotF1650, NotF1611 and NotF1581, and using as a template the plasmid pBluescript KS+/SmaI/F, in which the entire region for F protein had been inserted into pBluescript KS+ (Stratagene) at the SmaI site. The amplified fragments were digested with XhoI and NotI, and then each was inserted into the XhoI-NotI site of the plasmid that had been constructed from pCAGGS (Gene, vol.108, pp.193-200, 1991) by inserting an XhoI/NotI linker into the EcoRI site to construct plasmids (15 amino acid deletion: pCAGGS-Fct27; 28 amino acid deletion: pCAGGS-Fct14; 38 amino acid deletion: pCAGGS-Fct4).

[4] Construction of cytoplasmic domain-lacking F protein expression plasmid containing SIV cytoplasmic domain

Plasmids were constructed (Fig. 17) by adding the first 11 amino acids from 5' end of the SIV cytoplasmic domain (SIVct11) to cytoplasmic domain-lacking F protein expression plasmids (the numbers of amino acids in the cytoplasmic domain of F protein are the same as those in the plasmids prepared in [3]). Fragments corresponding to the above-described three types lacking the amino acids but containing the SIV cytoplasmic domain added were amplified by PCR using the pairs of primers XhFF and SA-F1650, and SA-F1611 and SA-F1581, and using as a template the plasmid pBluescript KS+/SmaI/F, in which the entire region for F protein had been inserted into pBluescript KS+ (Stratagene) at the SmaI site. The amplified fragments were digested with XhoI and NotI, and then each was inserted into the XhoI-NotI site of the plasmid that had been constructed from pCAGGS (Gene, vol.108, pp.193-200, 1991) by inserting an XhoI/NotI linker into the EcoRI site to construct plasmids (SIVct11 addition + 15 amino acid deletion: pCAGGS-Fct27/SIVct11; SIVct11 addition + 28 amino acid deletion: pCAGGS-Fct14/SIVct11; and SIVct11 addition + 38 amino acid deletion: pCAGGS-Fct4/SIVct11).

[Example 12] Preparation and performance analysis of Sendai virus envelope-pseudotyped lentivirus vector

<Cell culture>

293T cells (human fetal kidney cell line) were cultured in Dulbecco's Modified Eagle Medium(DMEM)-High glucose (Gibco BRL) containing 10% inactivated calf serum (BIO WHITTAKER) under 10% CO_2 at 37°C.

BEAS-2B cells (human epithelial cell line derived from the trachea) were cultured in a mixture of Minimum Essential Medium (MEM) (Gibco BRL) and RPMI1640 (1:1 ratio) containing 10% inactivated calf serum (BIO WHITTAKER) under 5% CO_2 at 37°C.

<Preparation of vector>

293T cells were plated in a 6-well plastic culture plate at a cell density of 5x 10^5 cells/well, and then incubated under 10% CO_2 at 37°C for 48 hours. The culture medium was changed with 800 μl/well of DMEM containing 1% bovine serum albumin. The cells were then used in transfection. In the combinations as indicated in Table 6 below, 1200 ng of gene transfer vector (pGL3C/CMVL.U3G2/RREc/s/CMVF EGFP/3LTRΔU3), 360 ng of packaging vector (pCAGGS/SIVaqm gag-tat/rev), Sendai virus HN, F protein expression plasmid pCAGGS-SIVct/HN, pCAGGS-SIVct+HN, pCAGGS-Fct4, pCAGGS-Fct14, pCAGGS-Fct27, pCAGGS-Fct4/SIVct11, pCAGGS-Fct14/SIVct11, and pCAGGS-Fct27/SIVct11 were dissolved in 100 μl of Opti MEM (Gibco BRL) in each well. Then, 6 μl of PLUS Reagent (Gibco BRL) was added to the well. The mixture was stirred, and then allowed to stand still at room temperature for 15 minutes. A solution obtained by diluting 4 μl of LIPOFECTAMINE Reagent (Gibco BRL) with 100 μl of Opti MEM was added to the mixture. The mixed solution was stirred, and then allowed to stand still at room temperature for another 15 minutes. The resulting mixture was added dropwise to the 293 T cells prepared above while being stirred gently. The cells were then incubated under 10% CO_2 at 37°C for 3 hours. 1 ml of DMEM containing 1% bovine serum albumin and 15 μg/ml trypsin (Gibco BRL) was added to each well. After culture under 10% CO_2 at 37°C for 16 to 24 hours, the culture medium in each well was changed with 2 ml of DMEM containing 1% bovine serum albumin and 7.5 μg/ml trypsin (Gibco BRL). After subsequently culturing for

24 hours, culture supernatant was collected. This supernatant was then filtered with a filter having a 0.45-μm diameter and the resulting solution was used as a vector solution.

Table 6

		HN protein expression plasmid		
		pCAGGS-HN	pCAGGS-SIVct/HN	pCAGGS-SIVct+HN
F protein expression plasmid	pCAGGS-Fct4	○	○	○
	pCAGGS-Fct14	○	○	○
	pCAGGS-Fct27	○	○	○
	PCAGGS-Fct4/SIVct11	○	○	○
	PCAGGS-Fct14/SIVct11	○	○	○
	PCAGGS-Fct27/SIVct11	○	○	○

<SIVagm vector-mediated gene transfer>

293T cells and BEAS-2B cells as target cells were plated in 6-well plastic culture plates at a cell density of 1x 10^6 cells/well, and incubated under 10% CO_2 at 37°C; the incubation was carried out for 48 hours for 293T cells or for 24 hours for BEAS-2B cells. Then, the culture medium was removed from the culture plates, and 1 ml of a mixture obtained by adding polybrene (Sigma) to the vector solution at a final concentration of 8 μg/ml was overlaid on the cells. The plates were incubated at 37°C for 3 hours to transfect the vector to the cells; the incubation was carried out under 10% CO_2 for 293T cells or under 5% CO_2 for BEAS-2B cells. After three hours, 1 ml of culture medium containing 20% inactivated calf serum (BIO WHITTAKER) was added to the cells, and the plates were incubated at 37°C for 48 hours; the incubation was carried out under 10% CO_2 for 293T cells or under 5% CO_2 for BEAS-2B cells.

<Vector titration>

The titer was determined based on the number of cells into which genes had been introduced using 1 ml of vector solution. Infection was carried out with 1 ml of the vector solution according to the method described above. 48 hours after infection, the cells were fixed with PBS (Invitrogen) containing 2% formaldehyde and 0.2% glutaraldehyde at room temperature for 20 minutes, and washed once

with PBS. The mean value for the number of cells containing the transferred gene in a visual field was determined from three different visual fields under a fluorescence invert microscope (DMIRB(SLR), Leica) with 200-fold magnification, and multiplied by the coefficient 854.865 that had been determined based on the area of the visual field and the area of the plate to determine the titer. The unit of titer was defined as Transducing Unit (T.U.)/ml.

<Large-scale preparation and enrichment of vector>

293T cells were plated in 15-cm plastic dishes at a cell density of 5×10^6 cells/dish, and incubated under 10% CO_2 at 37°C for 48 hours. The culture medium was changed with 10 ml/dish of DMEM containing 1% bovine serum albumin. The cells were used in transfection. 8 μg of gene transfer vector (pGL3C/CMVL.U3G2/RREc/s/CMVF LacZ/3LTRΔU3), 2.4 μg of packaging vector (pCAGGS/SIVagm gag-tat/rev), 1.6 μg each of Sendai virus HN protein expression plasmid pCAGGS-SIVct+HN and F protein expression plasmid pCAGGS-Fct4 were dissolved in 1.5 ml of Opti MEM(Gibco BRL) in each dish. Then, 40 μl of PLUS Reagent (Gibco BRL) was added to the well. The mixture was stirred, and then allowed to stand still at room temperature for 15 minutes. A solution obtained by diluting 60 μl of LIPOFECTAMINE (Gibco BRL) with 1.5 ml of Opti MEM was added to the mixture. The mixed solution was stirred, and then allowed to stand still at room temperature for another 15 minutes. The resulting mixture was added dropwise to the 293 T cells prepared above while being stirred gently. The cells were then incubated under 10% CO_2 at 37°C for 3 hours. 10 ml of DMEM containing 1% bovine serum albumin and 15 μg/ml trypsin (Gibco BRL) was added to each well. After culture under 10% CO_2 at 37°C for 16 to 24 hours, the culture medium in each dish was changed with 20 ml of DMEM containing 1% bovine serum albumin and 7.5 μg/ml trypsin (Gibco BRL). After subsequently culturing for 24 hours, culture supernatant was collected. This supernatant was then filtered with a filter having a 0.45-μm diameter. Then, the filtrate was centrifuged at 16,000x g (Beckman J-25I, JA-18) at 4°C for one hour. The pellet was dissolved in PBS (containing 5% FCS and 2 μg/ml polybrene). The resulting solution was stored at -80°C.

<Results>

The gene transfer vector (pGL3C/CMVL.U3G2/RREc/s/CMVF EGFP/3LTRΔU3), packaging vector (pCAGGS/SIVagm gag-tat/rev), Sendai virus HN protein expression plasmid (pCAGGS-SIVct/HN or pCAGGS-SIVct+HN), and F protein expression plasmid (pCAGGS-Fct4, pCAGGS-Fct14, pCAGGS-Fct27, pCAGGS-Fct4/SIVct11, pCAGGS-Fct14/SIVct11, pCAGGS-Fct27/SIVct11) were co-transfected in various combinations into cells. Then, genes were successfully introduced into 293T cells and BEAS-2B cells (Figs 18, 19, and 20). Gene transfer was achievable with the modified HN and F protein expression plasmids alone without co-expressing VSV-G. Thus, it was demonstrated that it was possible to provide SIVagm-based pseudotype lentivirus vector modified using Sendai virus F and HN proteins. The titer of the pseudotype vector toward 293T cells was approximately $3.6x$ 10^4 T.U./ml. The titer was highest when pCAGGS-Fct4 and Fct4/SIVct11 were used in combination as the F protein expression plasmid; among combinations of the two types of F protein expression plasmids with the three types of HN protein expression plasmids, the highest titer was achieved by the combined use of the HN protein expression plasmid pCAGGS-SIVct+HN.

It was tested whether the vector that achieved the highest titer, which had been prepared by co-transfecting F protein expression plasmid pCAGGS-Fct4 and HN protein expression plasmid pCAGGS-SIVct+HN, could be enriched by centrifugation. As a result, the F-HN pseudotype vector was confirmed to be enriched to a high level by centrifugation (Fig. 21).

<Tested oligonucleotides>

The synthetic oligonucleotides used, except SA-F1611 and SA-F1581, were synthesized and purified with reverse-phase cartridges or PAGE in the Biochemical Research Division of the Nippon Flour Mills Co., Ltd. via the Department of Custom DNA Synthesis, Greiner Japan. SA-F1611 and SA-F1581 were synthesized in Sawady Technology Co., and used after being purified by HPLC.

Xho+Xma:

5'-TCGAGATGTGGTCTGAGTTAAAAATCAGGAGCAACGACGGAGGTGAAGGACCAGACGCCAA CGACCC-3' (SEQ ID NO: 25)

Xma-Xho:

5'-CCGGGGGTCGTTGGCGTCTGGTCCTTCACCTCCGTCGTTGCTCCTGATTTTTAACTCAGAC

CACATC-3' (SEQ ID NO: 26)

    Xma+131:

5'-CCGGGGAAAGGGGGTGCAACACATCCATATCCAGCCATCTCTACCTGTTTATGGACAGA-3

' (SEQ ID NO: 27)

    135-Xma:

5'-ACCCTCTGTCCATAAACAGGTAGAGATGGCTGGATATGGATGTGTTGCACCCCCTTTCC-3

' (SEQ ID NO: 28)

    132+Bam:

5'-GGGTTAGGTGGTTGCTGATTCTCTCATTCACCCAGTGGG-3' (SEQ ID NO: 29)

    Bam-136:

5'-GATCCCCACTGGGTGAATGAGAGAATCAGCAACCACCTA-3' (SEQ ID NO: 30)

    FSIVhn:

5'-GAGACTCGAGATGTGGTCTGAGTTAAAAATCAGG-3' (SEQ ID NO: 31)

    RhnSIV:

5'-AGAGGTAGACCAGTACGAGTCACGTTTGCCCCTATCACCATCCCTAACCCTCTGTCCATAA

AC-3' (SEQ ID NO: 32)

    XhFF: 5'-CCGCTCGAGCATGACAGCATATATCCAGAGA-3' (SEQ ID NO: 33)

    NotF1650:

5'-ATAGTTTAGCGGCCGCTCATCTGATCTTCGGCTCTAATGT-3' (SEQ ID NO: 34)

    NotF1611:

5'-ATAGTTTAGCGGCCGCTCAACGGTCATCTGGATTACCCAT-3' (SEQ ID NO: 35)

    NotF1581:

5'-ATAGTTTAGCGGCCGCTCACCTTCTGAGTCTATAAAGCAC-3' (SEQ ID NO: 36)

    SA-F1650:

5'-ATAGTTTAGCGGCCGCCTATGGAGATAGAGGAACATATCCCTGCCTAACCCTTCTGATCTT

CGGCTCTAATGT-3' (SEQ ID NO: 37)

    SA-F1611:

5'-ATAGTTTAGCGGCCGCCTATGGAGATAGAGGAACATATCCCTGCCTAACCCTACGGTCATC

TGGATTACCCAT-3' (SEQ ID NO: 38)

    SA-F1581:

5'-ATAGTTTAGCGGCCGCCTATGGAGATAGAGGAACATATCCCTGCCTAACCCTCCTTCTGAG

TCTATAAAGCAC-3' (SEQ ID NO: 39)

[Example 13] Preparation and performance analysis of Sendai virus
envelope-pseudotyped retroviral vector

<Cell culture>

293T cells (human fetal kidney cell line) were cultured in Dulbecco's Modified Eagle Medium(DMEM)-High glucose (Gibco BRL) containing 10% inactivated calf serum (BIO WHITTAKER) under 10% CO_2

5   at 37°C.

<Preparation of vector>

293T cells were plated in a 6-well plastic culture plate at a cell density of 5x 10^5 cells/well, and then incubated under 10% CO_2 at 37°C for 48 hours. The culture medium was changed with 800 μl/well of DMEM

10 containing 1% bovine serum albumin. The cells were then used in transfection. In the combinations as indicated in Table 7 below, 700 ng of gene transfer vector pMSCV EGFP, 300 ng of ecotropic envelope and gag-pol expression plasmid (IMGENEX), 200 ng of Sendai virus F protein expression plasmid pCAGGS-Fct4, 200 ng each of HN protein

15 expression plasmid pCAGGS-HN, pCAGGS-SIVct/HN, and pCAGGS-SIVct+HN were dissolved in 100 μl of Opti MEM (Gibco BRL) in each well. Then, 6 μl of PLUS Reagent (Gibco BRL) was added to the well. The mixture was stirred, and then allowed to stand still at room temperature for 15 minutes. A solution obtained by diluting 4 μl of LIPOFECTAMINE

20 Reagent (Gibco BRL) with 100 μl of Opti MEM was added to the mixture. The mixed solution was stirred, and then allowed to stand still at room temperature for another 15 minutes. The resulting mixture was added dropwise to the 293 T cells prepared above while being stirred gently. The cells were then incubated under 10% CO_2 at 37°C for 3

25 hours. 1 ml of DMEM containing 1% bovine serum albumin and 15 μg/ml trypsin (Gibco BRL) was added to each well. After culture under 10% CO_2 at 37°C for 16 to 24 hours, the culture medium in each well was changed with 2 ml of DMEM containing 1% bovine serum albumin and 7.5 μg/ml trypsin (Gibco BRL). After the subsequent culture for 24 hours,

30 the culture supernatant was collected, and filtered with a filter with 0.45-μm diameter pores; the resulting solution was used as a vector solution.

Table 7

		HN protein expression plasmid		
		pCAGGS-HN	pCAGGS-SIVct/HN	pCAGGS-SIVct+HN
F protein expression plasmid	pCAGGS-Fct4	◯	◯	◯

<SIVagm vector-mediated gene transfer>

5   293T cells as target cells were plated in 6-well plastic culture plates at a cell density of 1x 10^5 cells/well, and incubated under 10% CO_2 at 37°C for 48 hours. Then, the culture medium was removed from the culture plates, and 1 ml of a mixture obtained by adding polybrene (Sigma) to the vector solution at a final concentration

10   of 8 μg/ml was overlaid on the cells. The plates were incubated under 10% CO_2 at 37°C for 3 hours to transfect the vector to the cells. After three hours, 1 ml of a culture medium containing 20% inactivated calf serum (BIO WHITTAKER) was added to the cells, followed by incubation under 10% CO_2 at 37°C for 48 hours.

15   <Vector titration>

The titer was determined based on the number of cells into which genes had been introduced using 1 ml of vector solution. Infection was carried out with 1 ml of the vector solution according to the method described above. 48 hours after infection, the cells were fixed

20   with PBS (Invitrogen) containing 2% formaldehyde and 0.2% glutaraldehyde at room temperature for 20 minutes, and washed once with PBS. The mean value for the number of cells containing the transferred gene in a visual field was determined from three different visual fields under a fluorescence invert microscope (DMIRB(SLR),

25   Leica) with 200-fold magnification, and multiplied by the coefficient 854.865 that had been determined based on the area of the visual field and the area of the plate to determine the titer. The unit of titer was defined as Transducing Unit (T.U.)/ml.

<Results>

30   The gene transfer vector pMSCV EGFP, ecotropic envelope and gag-pol expression plasmid, Sendai virus F protein expression plasmid pCAGGS-Fct4, HN protein expression plasmid pCAGGS-HN, pCAGGS-SIVct/HN, and pCAGGS-SIVct+HN were co-transfected in

combination to cells. Gene transfer into 293T cells was successfully achieved (Fig. 22). When the modified HN and F protein expression plasmids were used, genes were successfully introduced into human 293T cells, to which ecotropic envelope-containing viruses have no infectivity. Thus, it was demonstrated that it was possible to provide MSCV-based pseudotype retroviral vector modified using Sendai virus F and HN proteins. Further, pCAGGS-Fct4 was used as an F protein expression plasmid in combination with the three types of HN protein expression plasmids; the titer of pseudotype retroviral vector was highest (1.1×10^5 T.U./ml), when it was used in combination with the HN protein expression plasmid pCAGGS-SIVct+HN.

[Example 14] Production of VSV-G/HN pseudotype lentivirus vector and comparison with VSV-G pseudotype lentivirus vector for the efficiency of gene transfer into human bone marrow cells including hematopoietic stem cells

<Cell culture>

293T cells (human fetal kidney cell line) were cultured in Dulbecco's Modified Eagle Medium(DMEM)-High glucose (Gibco BRL) containing 10% inactivated calf serum (BIO WHITTAKER) under 10% CO_2 at 37°C.

<Vector titration>

The titer was determined based on the number of cells into which genes had been introduced using 1 ml of vector solution. Infection was carried out with 1 ml of the vector solution according to the method described above. 48 hours after infection, the cells were fixed with PBS (Invitrogen) containing 2% formaldehyde and 0.2% glutaraldehyde at room temperature for 20 minutes, and washed once with PBS. The mean value for the number of cells containing the transferred gene in a visual field was determined from three different visual fields under a fluorescence invert microscope (DMIRB(SLR), Leica) with 200-fold magnification, and multiplied by the coefficient 854.865 that had been determined based on the area of the visual field and the area of the plate to determine the titer. The unit of titer was defined as Transducing Unit (T.U.)/ml.

<Large-scale preparation and enrichment of vector>

293T cells were plated in 15-cm plastic dishes at a cell density of 5×10^6 calls/dish, and incubated under 10% CO_2 at 37°C for 48 hours. The culture medium was changed with 10 ml/dish of DMEM. The cells were used in transfection. 8 μg of gene transfer vector (pGL3C/CMVL.U3G2/RREc/s/CMVF EGFP/3LTRΔU3), 2.4 μg of packaging vector (pCAGGS/SIVagm gag-tat/rev), 1.6 μg each of VSV-G expression plasmid pVSV-G and Sendai virus HN protein expression plasmid pCAGGS-HN, pCAGGS-SIVct/HN, and pCAGGS-SIVct+HN were dissolved in combinations indicated in Table 8 below in 1.5 ml of Opti MEM(Gibco BRL) in each dish. Then, 40 μl of PLUS Reagent (Gibco BRL) was added to the well. The mixture was stirred, and then allowed to stand still at room temperature for 15 minutes. A solution obtained by diluting 60 μl of LIPOFECTAMINE (Gibco BRL) with 1.5 ml of Opti MEM was added to the mixture. The mixed solution was stirred, and then allowed to stand still at room temperature for another 15 minutes. The resulting mixture was added dropwise to the 293 T cells prepared above while being stirred gently. The cells were then incubated under 10% CO_2 at 37°C for 3 hours. 20 ml of DMEM containing 20% inactivated calf serum (BIO WHITTAKER) was added to each well. After culture under 10% CO_2 at 37°C for 16 to 24 hours, the culture medium in each dish was changed with 20 ml of DMEM containing inactivated calf serum (BIO WHITTAKER). After subsequently culturing for 24 hours, culture supernatant was collected. This supernatant was then filtered with a filter having a 0.45-μm diameter. The filtrate was centrifuged at 42,390x g (TOMY SRX-201, TA21BH) at 4°C for 90 minutes. The pellet was dissolved in a 1/100 volume of reverse transcription reaction solution (TBS, 10 mM $MgCl_2$, 3 mM SPERMINE, 0.3 nM SUPERMIDINE, 100 mM dNTPs). The solution was incubated at 37°C for two hours. After reverse transcription, the reaction was centrifuged at 42,390x g (TOMY SRX-201, TA21BH) at 4°C for two hours, and the pellet was dissolved in PBS (containing 5% FCS and 2 μg/ml polybrene). The resulting solution was stored at -80°C until used.

Table 8

	pCAGGS-HN	pCAGGS-SIVct/HN	pCAGGS-SIVct+HN
pVSV-G	◯	◯	◯

<Gene transfer into human bone marrow CD34$^+$ cells>

Human bone marrow CD34$^+$ cells were purchased from BIO WHITTAKER. After thawing, the cells were cultured in Iscove's modified DMEM (IMDM) containing 50 ng/ml IL-6, 100 ng/ml TPO, 100 ng/ml Stem Cell Factor (GIBCO BRL), 100 ng/ml Flt-3 ligand (Research Diagnostics, Flanders, NJ)(all human recombinants), and 10% FCS under 5% CO_2 at 37°C for 48 hours. After incubation, the medium was removed and the viral vector was added to 2x 10^5 cells using a virus solution of either 2x 10^6 or 10^7 T.U./ml. Then, 50 ng/ml IL-6, 100 ng/ml TPO, 100 ng/ml Stem Cell Factor (GIBCO BRL), and 100 ng/ml Flt-3 ligand were added to the cells. The cells were harvested 96 hours after the start of incubation.

< Flow cytometry analysis>

The harvested cells were stained using a PE-labeled anti-human CD34 antibody (Becton Dickinson), and then analyzed using two types of fluorescence signals corresponding to GFP and PE by flow cytometry (EPICS ELITE, Coulter).

<Results>

When the VSV-G pseudotype vector was used at m.o.i.=10, the proportion of GFP-positive cells in CD34$^+$ cells was 9.7%; with the pseudotype vectors VSV-G and HN, SIVct/HN and SIVct+HN, the proportion of GFP-positive cells in CD34$^+$ cells was 43.9, 25.2 and 19.7%, respectively (Table 9). On the other hand, using the VSV-G pseudotype vector at m.o.i.=50, the proportion of GFP-positive cells in CD34$^+$ cells was 51.4%; with VSV-G and HN, SIVct/HN and SIVct+HN pseudotype vectors, the proportions of GFP positive cells in CD34$^+$ cells were 43.0, 70.8, and 68.4%, respectively (Table 10).

Based on the above findings, it was confirmed that the pseudotype vector prepared by co-expressing VSV-G protein and HN protein ensured increased efficiency of gene transfer into human bone marrow cells including hematopoietic stem cells.

Table 9 Population of GFP-positive cell (%) at m.o.i.=10

	VSV-G	VSV-G/HN	VSV-G/SIVct/HN	VSV-G/SIVct+HN
CD34$^+$	9.7	43.9	25.2	19.7
CD34$^-$	16.7	8.0	0.3	0.2

Table 10 Population of GFP-positive cell (%) at m.o.i.=50

	VSV-G	VSV-G/HN	VSV-G/SIVct/HN	VSV-G/SIVct+HN
CD34⁺	51.4	43.0	70.8	68.4
CD34⁻	0.2	0.6	0.3	0.2

[Example 15] Construction of expression plasmid for influenza virus envelope protein

An expression plasmid was constructed, which encodes influenza virus (H1N1)-derived hemagglutinin protein (HA). A fragment was amplified by PCR using primers HAFNot and HARNot and plasmid pDREF HisD (Microbiol. Immunol., 44(8), 677-685, 2000) as a template. The amplified fragment was digested with NotI, and then inserted into the NotI site of the vector prepared by adding an XhoI-NotI site to pCAGGS (Gene, vol.108, pp.193-200, 1991). The plasmid obtained by the procedure described above was used as the HA protein expression plasmid pCAGGS-HA.

The synthetic oligonucleotides used were synthesized and purified with reverse-phase cartridges or PAGE in the Biochemical Research Division of the Nippon Flour Mills Co., Ltd. via the Department of Custom DNA Synthesis, Greiner Japan.

HAFNot: 5'-GAGAGCGGCCGCCCAAAATGAAGGCAAAACTACTG-3' (SEQ ID NO: 48)

HARNot: 5'-GATGCGGCCGCTCAGATGCATATTCTGCAC-3' (SEQ ID NO: 49)

[Example 16] Preparation and performance analysis of influenza virus envelope pseudotype lentivirus vector

<Cell culture>

293T cells (human fetal kidney cell line) were cultured in Dulbecco's Modified Eagle Medium(DMEM)-High glucose (Gibco BRL) containing 10% inactivated calf serum (BIO WHITTAKER) under 10% CO_2 at 37°C.

<Preparation of vector>

293T cells were plated in a 6-well plastic culture plate at a cell density of 5x 10^5 cells/well, and then incubated under 10% CO_2 at 37°C for 48 hours. The culture medium was changed with 800 µl/well of DMEM containing 1% bovine serum albumin. The cells were then used in

transfection. 1200 ng of gene transfer vector (pGL3C/CMVL.U3G2/RREc/s/CMVF EGFP/3LTRΔU3), 360 ng of packaging vector (pCAGGS/SIVagm gag-tat/rev), and 240 ng of HA protein expression plasmid pCAGGS-HA were dissolved in 100 μl of Opti MEM (Gibco BRL) in each well. Then, 6 μl of PLUS Reagent (Gibco BRL) was added to the well. The mixture was stirred, and then allowed to stand still at room temperature for 15 minutes. A solution obtained by diluting 4 μl of LIPOFECTAMINE Reagent (Gibco BRL) with 100 μl of Opti MEM was added to the mixture. The mixed solution was stirred, and then allowed to stand still at room temperature for another 15 minutes. The resulting mixture was added dropwise to the 293 T cells prepared above while being stirred gently. The cells were then incubated under 10% CO_2 at 37°C for 3 hours. 1 ml of DMEM containing 1% bovine serum albumin and 10 μg/ml trypsin (Gibco BRL) was added to each well. After culture under 10% CO_2 at 37°C for 16 to 24 hours, the culture medium in each well was changed with 2 ml of DMEM containing 1% bovine serum albumin, 5 μg/ml trypsin (Gibco BRL), and 50 units of neuraminidase (Roche). After subsequently culturing for 24 hours, culture supernatant was collected. This supernatant was then filtered with a filter having a 0.45-μm diameter and the resulting solution was used as a vector solution.

<SIVagm vector-mediated gene transfer>

293T cells as target cells were plated in 6-well plastic culture plates at a cell density of 1x 10^6 cells/well, and incubated under 10% CO_2 at 37°C for 48 hours. Then, the culture medium was removed from the culture plates, and 1 ml of a mixture obtained by adding polybrene (Sigma) to the vector solution at a final concentration of 8 μg/ml was overlaid on the cells. The plates were incubated under 10% CO_2 at 37°C for 3 hours to transfect the vector to the cells. After three hours, 1 ml of a culture medium containing 20% inactivated calf serum (BIO WHITTAKER) was added to the cells, and the cells were incubated under 10% CO_2 at 37°C for 48 hours.

<Vector titration>

The titer was determined based on the number of cells into which genes had been introduced using 1 ml of vector solution. Infection was carried out with 1 ml of the vector solution according to the

method described above. 48 hours after infection, the cells were fixed with PBS (Invitrogen) containing 2% formaldehyde and 0.2% glutaraldehyde at room temperature for 20 minutes, and washed once with PBS. The mean value for the number of cells containing the

5 transferred gene in a visual field was determined from three different visual fields under a fluorescence invert microscope (DMIRB (SLR), Leica) with 200-fold magnification, and multiplied by the coefficient 854.865 that had been determined based on the area of the visual field and the area of the plate to determine the titer. The unit of titer

10 was defined as Transducing Unit (T.U.)/ml.

<Large-scale preparation and enrichment of vector>

293T cells were plated in 15-cm plastic dishes at a cell density of 5×10^6 calls/dish, and incubated under 10% CO_2 at 37°C for 48 hours. The culture medium was changed with10 ml/dish of DMEM containing 1%

15 bovine serum albumin. The cells were used in transfection. 8 µg of gene transfer vector (pGL3C/CMVL.U3G2/RREc/s/CMVF LacZ/3LTRΔU3), 2.4 µg of packaging vector (pCAGGS/SIVagm gag-tat/rev), and 1.6 µg of HA protein expression plasmid pCAGGS-HA were dissolved in 1.5 ml of Opti MEM(Gibco BRL) in each dish. Then, 40 µl of PLUS Reagent (Gibco

20 BRL) was added to the well. The mixture was stirred, and then allowed to stand still at room temperature for 15 minutes. A solution obtained by diluting 60 µl of LIPOFECTAMINE (Gibco BRL) with 1.5 ml of Opti MEM was added to the mixture. The mixed solution was stirred, and then allowed to stand still at room temperature for another 15 minutes.

25 The resulting mixture was added dropwise to the 293 T cells prepared above while being stirred gently. The cells were then incubated under 10% CO_2 at 37°C for 3 hours. 10 ml of DMEM containing 1% bovine serum albumin and 10 µg/ml trypsin (Gibco BRL) was added to each dish. After culture under 10% CO_2 at 37°C for 16 to 24 hours, the culture medium

30 in each dish was changed with 20 ml of DMEM containing 1% bovine serum albumin, 5 µg/ml trypsin (Gibco BRL), and 500 unit of neuraminidase (Roche). After the subsequent culture for 24 hours, the culture supernatant was collected, and filtered with a filter with 0.45-µm diameter pores. The filtrate was centrifuged at 16,000x g (Beckman

35 J-25I, JA-18) at 4°C for one hour. The pellet was dissolved in PBS (containing 5% FCS and 2 µg/ml polybrene). The resulting solution

was stored at -80°C.

<Results>

The gene transfer vector (pGL3C/CMVL.U3G2/RREc/s/CMVF
EGFP/3LTRΔU3), packaging vector (pCAGGS/SIVagm gag-tat/rev), and HA
5   protein expression plasmid (pCAGGS-HA) were co-transfected in various
combinations into cells. Then, genes were successfully introduced
into 293T cells (Fig. 23). Gene transfer was achievable without
co-expressing VSV-G. Thus, it was demonstrated that it was possible
to provide SIVagm-based pseudotype lentivirus vector using influenza
10   virus HA protein. The titer of the pseudotype vector toward 293T cells
was 1.3×10^4 T.U./ml. Further, it was tested whether the vector
prepared by the procedure described above could be enriched by
centrifugation. As a result, the HA pseudotype vector was confirmed
to be enriched to a high level by centrifugation (Fig. 24).

15

[Example 17] Preparation and performance analysis of lentivirus vector
pseudotyped by influenza virus and Sendai virus envelope

<Cell culture>

293T cells (human fetal kidney cell line) were cultured in
20   Dulbecco's Modified Eagle Medium(DMEM)-High glucose (Gibco BRL)
containing 10% inactivated calf serum (BIO WHITTAKER) under 10% CO_2
at 37°C.

<Vector preparation>

293T cells were plated in a 6-well plastic culture plate at a cell
25   density of 5×10^5 cells/well, and then incubated under 10% CO_2 at 37°C
for 48 hours. The culture medium was changed with 800 µl/well of DMEM
containing 1% bovine serum albumin. The cells were then used in
transfection. In the combinations as indicated in Table 11 below,
1200 ng of gene transfer vector (pGL3C/CMVL.U3G2/RREc/s/CMVF
30   EGFP/3LTRΔU3), 360 ng of packaging vector (pCAGGS/SIVagm
gag-tat/rev), and 240 ng each of Sendai virus HN protein expression
plasmid pCAGGS-HN, pCAGGS-SIVct/HN, pCAGGS-SIVct+HN and HA protein
expression plasmid were dissolved in 100 µl of Opti MEM (Gibco BRL)
in each well. Then, 6 µl of PLUS Reagent (Gibco BRL) was added to
35   the well. The mixture was stirred, and then allowed to stand still
at room temperature for 15 minutes. A solution obtained by diluting

4 µl of LIPOFECTAMINE Reagent (Gibco BRL) with 100 µl of Opti MEM was added to the mixture. The mixed solution was stirred, and then allowed to stand still at room temperature for another 15 minutes. The resulting mixture was added dropwise to the 293 T cells prepared above while being stirred gently. The cells were then incubated under 10% CO_2 at 37°C for 3 hours. 1 ml of DMEM containing 1% bovine serum albumin and 10 µg/ml trypsin (Gibco BRL) was added to each well. After culture under 10% CO_2 at 37°C for 16 to 24 hours, the culture medium in each well was changed with 2 ml of DMEM containing 1% bovine serum albumin, 5 µg/ml trypsin (Gibco BRL), and 50 units of neuraminidase (Roche). After the subsequent culture for 24 hours, the culture supernatant was collected, and filtered with a filter with 0.45-µm diameter pores; the resulting solution was used as a vector solution.

Table 11

		HN protein expression plasmid		
		pCAGGS-HN	pCAGGS-SIVct/HN	pCAGGS-SIVct+HN
HA protein expression plasmid	pCAGGS-HA	◯	◯	◯

<Results>

The gene transfer vector (pGL3C/CMVL.U3G2/RREc/s/CMVF EGFP/3LTRΔU3), packaging vector (pCAGGS/SIVagm gag-tat/rev), HA protein expression plasmid (pCAGGS-HA), and Sendai virus HN protein expression plasmid (pCAGGS-HN, pCAGGS-SIVct/HN, pCAGGS-SIVct+HN) were co-transfected in various combinations into cells. Then, genes were successfully introduced into 293T cells (Fig. 25). The neuraminidase is responsible for the cleavage of the linkage with sialic acid in the budding of influenza virus, and therefore was required for the preparation of the HA pseudotype. Thus, various HN expression plasmids were tested for the neuraminidase activity of Sendai virus HN protein; coexistence of HN protein led to the production of the desired vector. The result demonstrated that it was possible to provide novel HA/HN pseudotype lentivirus vector.

Industrial Applicability

The present invention provides retroviral vectors pseudotyped by

membrane proteins having hemagglutinin activity. The vectors of the present invention can preferably be used for gene therapy, etc. In particular, the vectors are useful for *in vivo* administration to the airways and for *ex vivo* administration to target hematopoietic stem cells.

CLAIMS

1. A substantially pure pseudotype retroviral vector that comprises a membrane protein having hemagglutinin activity.

2. The pseudotype retroviral vector according to claim 1, wherein the membrane protein having hemagglutinin activity is derived from a protein having hemagglutinin activity which is contained in a single-stranded negative strand RNA virus.

3. The pseudotype retroviral vector according to claim 2, wherein the membrane protein having hemagglutinin activity is derived from HN protein of paramyxovirus and/or HA protein of orthomyxovirus.

4. The pseudotype retroviral vector according to claim 2, wherein the membrane protein having hemagglutinin activity is a portion or the whole of a cytoplasmic domain of a protein having hemagglutinin activity which is contained in a single-stranded negative strand RNA virus, and wherein the portion or the whole has been modified by substitution, deletion, and/or addition.

5. The pseudotype retroviral vector according to any one of claims 1 to 3, further comprising F protein of paramyxovirus.

6. The pseudotype retroviral vector according to claim 5, wherein a portion or the whole of a cytoplasmic domain of the F protein has been modified by deletion and/or addition.

7. The pseudotype retroviral vector according to any one of claims 1 to 6, further comprising an envelope protein derived from a virus that is infectious to human cells.

8. The pseudotype retroviral vector according to claim 7, wherein the vector comprises an amphotropic envelope protein derived from a retroviral.

9. The pseudotype retroviral vector according to claim 7 or 8, wherein the vector comprises VSV-G protein derived from a vesicular stomatitis virus.

5   10. The pseudotype retroviral vector according to any one of claims 1 to 9, wherein the retroviral vector is derived from oncovirus.

11. The pseudotype retroviral vector according to any one of claims 1 to 9, wherein the retroviral vector is derived from lentivirus..

10

12. The pseudotype retroviral vector according to claim 11, wherein the lentivirus is derived from simian immunodeficiency virus.

13. The pseudotype retroviral vector according to claim 3 or 5,

15   wherein the paramyxovirus is Sendai virus.

14. The pseudotype retroviral vector according to any one of claims 1 to 13, wherein the vector comprises a foreign gene in an expressible manner.

20

15. The pseudotype retroviral vector according to claim 14, wherein the vector is used for transferring genes into a cell having mucus.

16. The pseudotype retroviral vector according to claim 15, wherein

25   the cell having mucus is a mucosal epithelial cell.

17. The pseudotype retroviral vector according to claim 16, wherein the mucosal epithelial cell is a mucosal epithelial cell of nasal cavity or pulmonary bronchial tube.

30

18. The pseudotype retroviral vector according to claim 14, wherein the vector is used for transferring genes into a hemocyte or hematopoietic cell.

35   19. The pseudotype retroviral vector according to claim 18, wherein the hemocyte or hematopoietic cell is a hematopoietic stem cell.

20. A composition for gene transfer, the composition comprising the pseudotype retroviral vector according to any one of claims 14 to 19.

21. The composition according to claim 20, wherein the composition is a pharmaceutical composition.

22. A method for introducing a foreign gene into cells, the method comprising the step of contacting cells with the pseudotype retroviral vector according to any one of claims 14 to 19.

23. A packaging cell for producing the pseudotype retroviral vector according to any one of claims 1 to 19, the cell comprising, in an expressible manner, a DNA encoding a protein having hemagglutinin activity.

24. A method for producing the pseudotype retroviral vector according to any one of claims 1 to 19, the method comprising the step of transcribing a retrovirus-derived gene transfer vector DNA in the packaging cell according to claim 23.

ABSTRACT

The present invention provides a retroviral vector containing a membrane protein having a hemagglutinin activity. The present inventors constructed a retroviral vector pseudotyped by the membrane protein having a hemagglutinin activity. This viral vector showed gene transfer at a high efficiency into host cells. In particular, it was established that genes can be transferred thereby at a high efficiency into cells into which genes can hardly be transferred by the conventional techniques, for example, blood cells and hematopoietic cells including hematopoietic stem cells, and mucous cells including mucosa epithelial cells. The viral vector of the present invention is highly useful as a vector for gene therapy.